

**The Effects of Sexually Transmitted Infections on the
Biological Correlates of HIV-1 Transmission and
Pathogenesis in Homosexual Men**

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To my parents
Suraya Sadiq and Matiullah Sadiq

And to the memory of my friend,
Abdul Majid Razvi (1950 -2001)

“It cannot be seen, cannot be felt
Cannot be heard, cannot be smelt...
It comes first and follows after,
Ends life, kills laughter.”

“A box without hinges, key or lid
Yet golden treasure inside is hid.”

The Hobbit
J R R Tolkien

ABSTRACT

In men, the concentration of HIV-1 RNA in semen is an important determinant of HIV-1 infectivity. In sub-Saharan Africa, urethritis has been associated with increases in seminal plasma HIV-1 RNA load in those not receiving antiretroviral therapy. Less is known of the impact of urethritis both within the developed world and in those receiving antiretroviral therapy. In-vitro evidence also suggests that syphilis may stimulate HIV-1 replication, potentially impacting on HIV-1 disease progression as well as its transmission. In this thesis I present a series of studies that examine the effects of urethritis (due to gonorrhoea, chlamydia or NSU), early syphilis and their treatments on HIV-1 RNA loads in blood and semen in homosexual men in the UK. The impact of urethritis on the presence of drug-resistant HIV-1 mutants found in semen among those receiving antiretroviral therapy was also analysed. In addition, effects on CD4 counts were examined in those with early syphilis. Finally a study on the feasibility of research involving semen donation is presented. The research demonstrates that gonococcal and chlamydial urethritis, but not NSU, increase HIV-1 RNA load in semen. In comparison with the increases previously demonstrated in Africa, the relative effects were similar but the absolute increases were smaller. Minimal effect was seen in those on suppressive antiretroviral therapy but among those on poorly suppressive antiretroviral regimes and those with gonococcal infection HIV-1 RNA loads in semen were high. In these patients multi-drug resistant HIV-1 was found in both genital and systemic compartments. The studies also demonstrate very little effect of early syphilis on either blood or semen plasma viral loads. However, early syphilis, particularly early latent syphilis, was associated with a reduction in CD4 count that was reversible on syphilis treatment. Studies involving semen donation in symptomatic homosexual men were shown to be feasible.

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ABBREVIATIONS

AIDS	Acquired Immune deficiency syndrome
ART	Antiretroviral therapy
BPVL	Blood plasma HIV-1 RNA load
CART	Combination antiretroviral therapy
cDNA	Complementary DNA
CRF	Circulating recombinant form
CU	Chlamydial urethritis
CTL	Cytotoxic T-lymphocyte
DNA	Deoxyribose nucleic acid
dNTP	Deoxynucleoside triphosphate
ELONA	Enzyme-linked oligonucleotide assay
GAGs	Glycosaminoglycans
GEE	Generalised estimated equations
GU	Gonococcal urethritis
GUD	Genital ulcer disease
GuSCN	Guanidinium thiocyanate
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
IL	Interleukin
LTR	Long terminal repeat
NASBA	Nucleic acid sequenced based amplification
NFAT	Nuclear factor of activated T cells
NF κ B	Nuclear factor kappa B
NGU	Non gonococcal urethritis

NSU	Non specific urethritis
PCR	Polymerase chain reaction
POD	Period of disease
RNA	Ribose nucleic acid
RPR	Rapid plasma reagin test
r <i>Tth</i> pol	recombinant <i>Thermus thermophilus</i> DNA polymerase
RT	Reverse transcriptase
RT-QPCR	Reverse-transcribed quantitative PCR
SPVL	Seminal plasma HIV-1 RNA load
STI	Sexually transmitted infection
TNF	Tumour necrosis factor

AIMS OF THIS THESIS

1. To examine the effects of urethritis (gonococcal, chlamydial and non-specific) and its treatment on the concentration of HIV-1 RNA in blood and semen plasma in patients receiving and not receiving antiretroviral therapy.
2. To examine the effects of early syphilis and its treatment on blood and semen HIV-1 RNA loads and on CD4 counts.
3. To examine the feasibility of studies involving semen donation among men with symptomatic urethritis.

CHAPTER 1. INTRODUCTION

1.1 Human Immunodeficiency Virus Infection

In the early 1980s reports of profound immune deficiency, among previously healthy individuals in the USA and Europe, amounted to the earliest descriptions of the acquired immune deficiency syndrome (AIDS) (Gottlieb et al 1981). It is estimated that by December 2003 more than 40 million people were living with AIDS or infection with Human immunodeficiency virus (HIV, the virus that causes AIDS) worldwide. Of these, 37 million were adults and over 2 million, children. The major burden of disease has fallen on the developing world, particularly sub-Saharan Africa (see Table 1.1) and the major mode of transmission is through heterosexual intercourse. In contrast, in developed countries, particularly in North America and Western Europe, although the number of heterosexual men and women living with HIV infection continues to increase, men who have sex with men still account for a significant proportion of new incident HIV infections. Other important modes of transmission globally include peri-natal mother to child transmission, transfusion of unscreened blood products and sharing of equipment used for intravenous drug abuse. Controversially iatrogenic transmission in developing countries has also been proposed (and rebutted) as an important cause of the pandemic (Gisselquist et al 2003; Schmid et al 2004). As the predominant mode of transmission is sexual, semen is likely to be a major vector of transmission. In this introduction I will outline the virology of HIV infection and explain what is known about the shedding of HIV in semen. The studies described in this thesis shed new light on our understanding of this biology.

Table 1.1 Regional HIV/AIDS statistics and features, end of 2003

(Data from UNAIDS: http://www.unaids.org/html/pub/Topics/Epidemiology/Slides02/Slide02_EP/core2003_en_ppt.pp)

	Adults and children living with HIV/AIDS (millions)	Adults and children newly infected with HIV (millions)	Adult prevalence rate [%]	Adult and child deaths due to AIDS (millions)
Sub-Saharan Africa	25.0 – 28.2	3.0 – 3.4	7.5 – 8.5	2.2 – 2.4
North Africa & Middle East	0.47 – 0.73	0.043 – 0.067	0.2 – 0.4	0.035 – 0.050
South and South-East Asia	4.6 – 8.2	0.61 – 1.1	0.4 – 0.8	0.33 – 0.59
East Asia and Pacific	0.7 – 1.3	0.15 – 0.27	0.1 – 0.1	0.032 – 0.058
Latin America	1.3 – 1.9	0.12 – 0.18	0.5 – 0.7	0.049 – 0.07
Caribbean	0.35 – 0.59	0.045 – 0.080	1.9 – 3.1	0.03 – 0.05
Eastern Europe & Central Asia	1.2 – 1.8	0.18 – 0.28	0.5 – 0.9	0.023 – 0.037
Western Europe	0.52 – 0.68	0.03 – 0.04	0.3 – 0.3	0.0026 – 0.0034
North America	0.79 – 1.2	0.036 – 0.054	0.5 – 0.7	0.012 – 0.018
Australia & New Zealand	0.012 – 0.018	0.0007 – 0.001	0.1 – 0.1	< 0.0001
Total	40 (34 – 46)	5 (4.2 – 5.8)	1.1 (0.9 – 1.3)	3 (2.5 – 3.5)

The ranges around the estimates in this table define the boundaries within which the actual numbers lie, based on the best available information. These ranges are more precise than those of previous years, and work is under way to increase even further the precision of the estimates that will be published mid-2004.

1.2 The Virus

HIV, first identified in 1983 (Barre-sinoussi et al 1983) is a member of the lentivirus genus (Family Retroviridae), containing only ribonucleic acid (RNA) and as such is unable to replicate outside of living host cells (Gallo et al 1984). Two major types of HIV exist. HIV-1, responsible for the majority of the pandemic, is itself classified into a number of sub-types phylogenetically. HIV-2, less transmissible than HIV-1, is found mainly in West African populations.

1.2.1 Structure of HIV-1 (see Figures 1.1 & 1.2)

HIV-1 consists of nine genes and 15 proteins. The mature virus consists of an electron dense core containing the viral genome (two short strands of RNA about 9200 nucleotide bases long), and the enzymes reverse transcriptase (RT), protease, ribonuclease, and integrase (Alizon et al 1984; Wain-Hobson et al 1985). The viral envelope, derived from host cell membrane, is studded with the glycoprotein gp120, which aids binding of virus to CD4 molecules that are found on some T-cell subsets and macrophages (Dalglish et al 1984; Klatzmann et al 1984; Klatzmann et al 1990). Virions are approximately 90 to 100 nm, in diameter.

The genome contains three major genes (gag, pol, and env) and accessory genes, important for control of viral replication and pathogenicity (Figure 1.1). Env encodes the envelope glycoproteins, gp120 and gp41 (both derived from the glycoprotein precursor gp160) and gag codes core proteins for example p24 (capsid, or "core" antigen), p17 (matrix), and p7 (nucleocapsid). The proteins coded by pol are the enzymes reverse transcriptase, protease and integrase (Figure 1.2).

Figure 1.1. The HIV-1 genome.

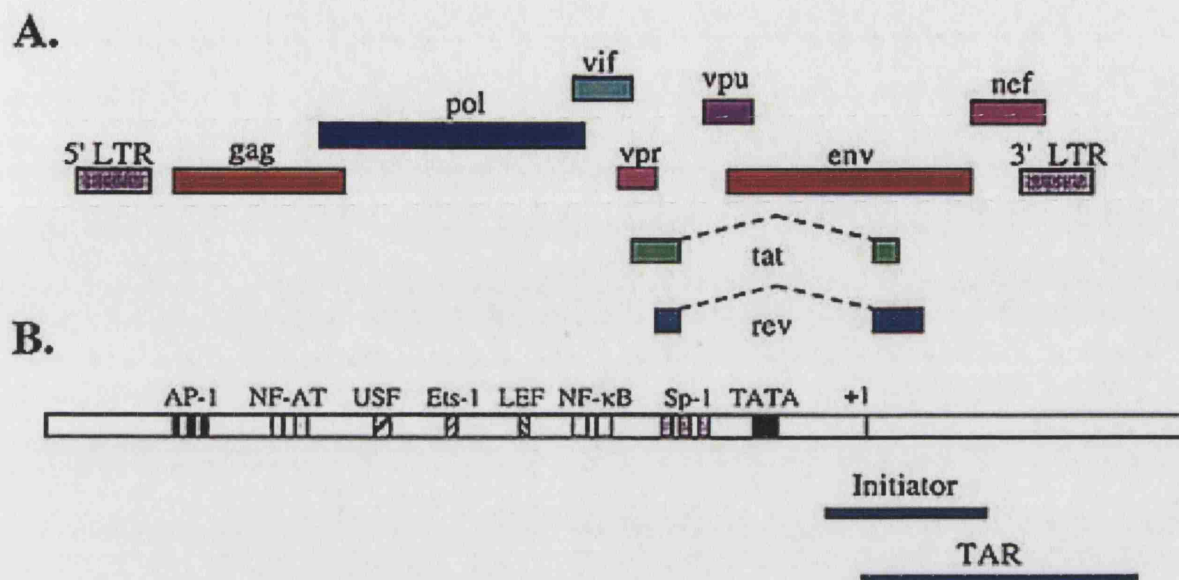


Diagram adapted from: www.bioscience.org/1997/v2/d/gottfred/3.htm (last accessed 18/05/04)

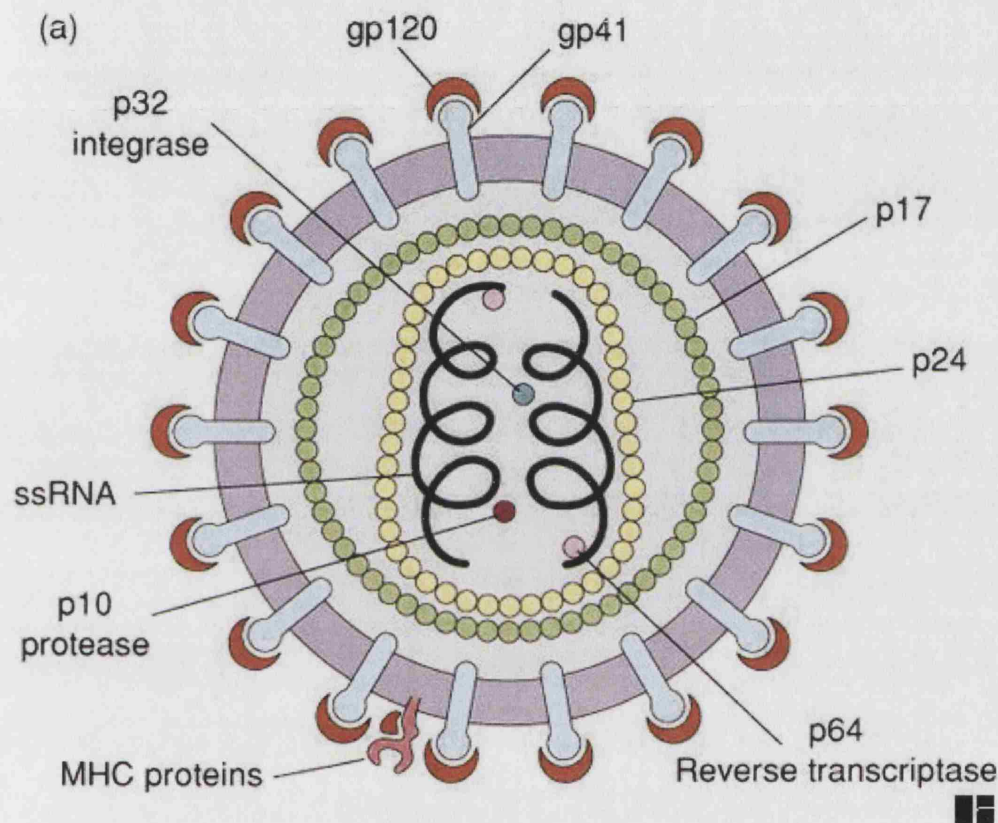
A. HIV-1 gene products encoded by the HIV-1 genomic sequence.

B. A representation of the HIV-1 promoter, which is located within the 5' LTR of the viral genome (see text). The core promoter consists of the initiator region, the TATA box, and three sites for binding the cellular transcription factor termed Sp1. An upstream enhancer contains binding sites for several cellular transcription factors including nuclear factor kappa B (NF-kB), lymphoid enhancer binding factor 1 (LEF), Ets-1 (a sub-group of the Ets family of transcription proteins), upstream stimulatory factor (USF), nuclear factor of activated T-cells (NF-AT), and activator protein 1 (AP-1).

Figure 1.2 Structure of HIV-1

Diagram from <http://www.whfreeman.com/immunology/CH22/figure22-02a.jpg>

(last accessed 18/05/04)



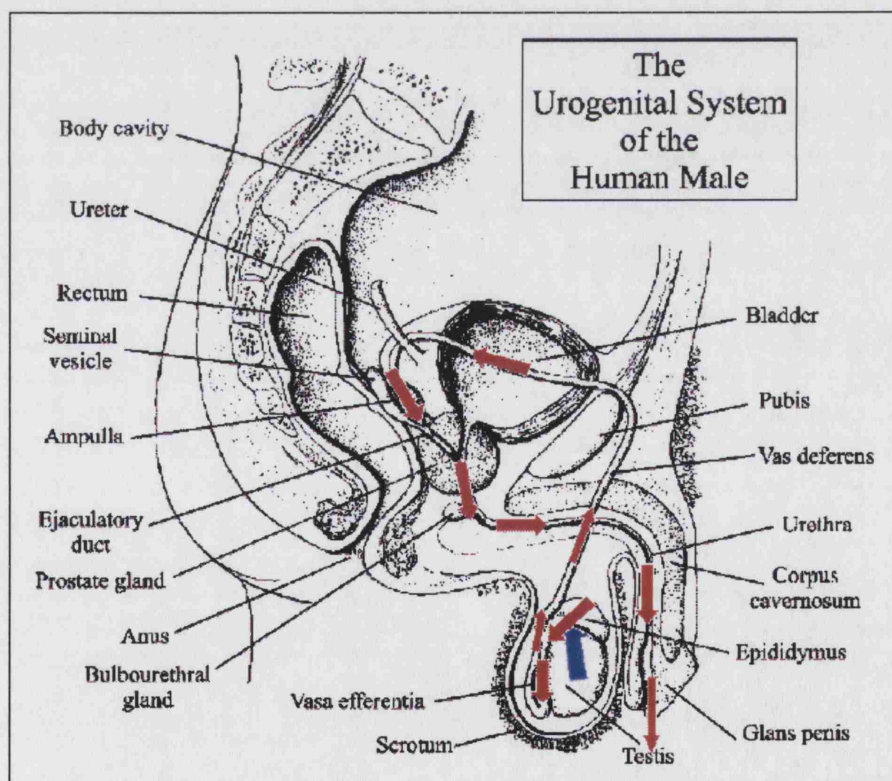
1.2.2 Life cycle

HIV-1 infects target cells through binding of gp120 to the CD4 receptor and this interaction is perhaps enhanced by glycosaminoglycans (GAGs), particularly heparin sulphate proteoglycan (Chang et al 2002; Trkola et al 1999; Ugolini et al 1999). GAGs are large negatively charged polysaccharides with repeating dissacharide units found in the extra-cellular matrix and on cell surfaces. The subsequent conformational change of gp120, unmasks amino acid sequences present in its hypervariable V3 loop region which then interact with a chemokine co-receptor,

duct of the seminal vesicle and continuing to the prostatic urethra. The prostate, a complex of tubular glands embedded in a thick fibromuscular stroma, surrounds the urethra just below the bladder. Its secretions contribute to seminal fluid. The bulbourethral glands are two pea-sized, lobulated bodies, below the prostate and behind and lateral to the urethra. The excretory duct of each gland, nearly 2.5 cm. long, opens by a minute orifice on the floor of the urethra. Each gland is made up of several lobules, held together by a fibrous coat. The bulbo-urethral glands produce the pre-ejaculatory fluid, and an alkaline fluid contribution to the semen that may serve to neutralize the acidic environment of the urethra, thereby protecting sperm.

Figure 1.3 The Male genital tract

(from <http://www.erin.utoronto.ca/~w3bio380/Lectsked/Lect05/Sperm1.htm> last accessed 05/05/04)



The arrows demonstrate the course of spermatozoa generated in the testes through the genital tract.

either CCR5, found predominantly on macrophages, or CXCR4, found predominantly on T-lymphocytes (Paxton et al 1998a). Tropism of HIV-1 for these different cells is largely determined by the tropism of virus for these co-receptors. In blood plasma, R5 virus (that which binds to CCR5) tends to be dominant in early HIV infection, is more likely to be transmitted during sexual transmission and is probably less pathogenic than lymphotropic virus X4 virus (that which binds to CXCR4). X4 virus becomes dominant in blood plasma as HIV disease advances and causes giant cell formation and fusion of lymphocytes in cell culture. Following the interaction between gp120 and the co-receptor, a resulting conformational change of gp41 leads to fusion of viral and cell membranes and injection of viral contents into the host cell cytoplasm.

Within the cell cytoplasm HIV-1 RNA is reversely transcribed by RT into linear and circular forms of complementary DNA (cDNA). Only linear forms are integrated into the host genome by the action of integrase (Sodeik 2002). Following integration, several inducible cellular transcription factors including SP-1, AP-1, nuclear Factor kappa B (NF- κ B) and nuclear factor of activated T-cells (NFAT) (Pereira et al 2000) regulate viral gene-expression through activation of DNA recognition sequences present at the 5' end of the viral genome, named the 5-long terminal repeat (LTR) and without such activation, provirus has the potential to remain latent for the entire lifespan of the infected cell (see figure 1.1). Transcription factors enter the nucleus following their release from inhibitory protein complexes within cytoplasm. This release is mediated by various signals including the pro-inflammatory factors tumour necrosis factor- α (TNF- α), interleukin-1 (IL-1) and lipopolysaccharide (Pomerantz et al 1990; Rabson et al 2000). TNF- α induces HIV-1 replication through a NF- κ B pathway and IL-1 and IL-6 are thought to enhance the effect of TNF- α (Lawn et al 2000a).

A number of proteins encoded by accessory HIV genes have important roles in enhancing replication and infectivity of virus. HIV transcription is enhanced through a positive feedback mechanism by one of the earliest HIV gene products, the viral protein Tat (Gatignol et al 2000), the RNA binding site of which is found on the LTR. Rev has a central role in moving viral RNA transcripts from the nucleus back to the cytoplasm for translation into viral proteins (Emerman et al 1998). Both Rev and Tat are essential for viral replication. Nef down-regulates cell surface levels of CD4 and MHC class I molecules and increases infectivity of new progeny virions as do Vif, Vpu, and Vpr (Stevenson 2003).

Gag, synthesized as a polypeptide, is cleaved by protease into several components. Viral proteins and genomic RNA are then assembled into mature virions in the inner aspect of the plasma membrane, mostly in so-called lipid rafts, areas rich in glycolipids, cholesterol and protein. Virus budding and release is controlled by numerous host cell proteins and differs between macrophages and lymphocytes. In macrophages virions accumulate in intracellular vesicles in addition to plasma membrane prior to release. Apart from CD4⁺ T-lymphocytes and macrophages, only astrocytes among CD4-negative cells have been found infected *in vivo* and infectable *in vitro* via a yet unidentified CD4-independent mechanism of infection (Brack-Werner 1999).

1.2.3 Diversity

RNA-dependent DNA synthesis lacks proof-reading activity and polymerisation errors and mutations in HIV-1 provirus are introduced approximately once per 2000 incorporated nucleotides. This high mutation rate leads to the rapid selection of HIV variants that can resist immune attack, are more cytotoxic or can

resist drug therapy. Over time, different tissues of the body may harbour different strains of HIV. The high mutation rate has also led to the emergence of many genetic forms of HIV-1, classified according to how their genetic sequences cluster together. Currently HIV-1 is classified into three phylogenetic groups, namely M (main), O (outlier) and N (non-M, non-O), perhaps reflecting independent introductions into the human population from chimpanzees. Most HIV-1 infections world-wide are a result of infection with group M. Group O represents a minority of infections, restricted to the West African country of Cameroon as does group N. Group M contains nine subtypes identified by the letters A-D, F-H, J and K, which have a geographic distribution. For example subtype B is found predominantly in Western European and North and South American HIV-1 epidemics, whereas subtypes A, C and D are found predominantly in Africa and South Asia. Additionally, a number of strains of virus have been identified which cluster differently depending on the area of the genome examined. These circulating recombinant forms (CRFs) have originated as a result of recombination of viruses following infection with two different subtypes. An example of this is CRF_AE (formerly named subtype E), thought to be an inter-subtype recombinant of subtypes A found in Africa and an as yet unidentified subtype E (Thomson et al 2002).

1.3 Pathogenesis of HIV disease

HIV infection brings about disease in humans primarily by its effects on immune depletion and dysfunction. Over time, gradual reduction of the plasma concentration of CD4⁺ T-lymphocytes is associated with vulnerability to opportunistic infections and malignancies, the AIDS defining illnesses. These illnesses are the principle causes of HIV-related death.

Soon after infection with HIV-1, the virus disseminates through plasma and body tissues reaching peak viraemia within a few weeks. This phase may be characterised by the so-called sero-conversion illness and is immunologically associated with temporary (and occasionally sustained) immune depletion and fall in the concentration of CD4⁺ T-lymphocytes. Most major HIV viral proteins are highly immunogenic and may produce weakly neutralising antibody responses for primary HIV-1 isolates in-vitro but these responses are not protective, failing to control viral replication in-vivo (Jacobson 1998; Montefiori et al 1996; Moog et al 1997; Moore et al 1995; Poignard et al 1999). More importantly, the development of a strong HIV-specific cytotoxic T-lymphocyte (CTL) response usually results in partial control of viral replication and some immune recovery (Borrow et al 1994; Borrow et al 1997; Pantaleo et al 1994; Schmitz et al 1999). Without this CTL response, HIV disease progression is rapid and relentless (Jin et al 1999; Koup et al 1994). However, even in the presence of a strong CTL response, perhaps billions of virions and millions of CD4⁺ T-cells are produced and destroyed daily (Ho et al 1995a). The subsequent generation of escape viral mutants, evading the ever evolving and chasing immune response plays an important part in disease progression during both primary (Borrow et al 1997; Price et al 1997) and chronic HIV infection (Goulder et al 1997; Phillips et al 1991). HIV-specific CD4⁺ T-lymphocytes are also important for disease progression and the degree to which they are impaired during primary infection may be critical (Rosenberg et al 1997).

The majority of AIDS defining illnesses occur as the CD4⁺ T-lymphocyte count falls below 200 x 10⁶ cells per cubic millimetre. In addition to the CD4⁺ T-lymphocyte count, the concentration of plasma HIV-1 RNA, or viral load, is an important prognostic marker predicting the likelihood of progression to AIDS or death

(Lyles et al 2000; O'Brien et al 1996). Potent combination antiretroviral therapies have had a significant effect on mortality and morbidity of HIV related disease, reversing immune depletion and raising CD4⁺ T-lymphocyte counts. In the absence of these therapies, the median time to death or AIDS has been estimated to be between 10 to 12 years in the developed world (Pantaleo et al 1993b). However this may vary from a matter of months to indefinitely as in the case of the so-called long term non-progressors (LTNPs). These individuals, approximately five percent of those HIV infected, maintain high CD4⁺ T-lymphocyte counts and low plasma HIV-1 RNA concentrations for long periods, have strong HIV-specific CTL activity and may have higher levels of HIV specific CD4⁺ T-lymphocyte responses compared with those who progress to AIDS (Norris et al 2002).

1.3.1 Host genetics

HIV disease progression is also dependent on individual genetic predisposition. Molecules of the major histocompatibility complex (MHC) class I haplotypes of infected individuals bind and present viral fragments to initiate immune responses and thus the efficiency of this antigen presentation is in part genetically pre-determined. For example HLA-A2, HLA-B27 and HLA-B57 haplotypes are associated with better clinical outcomes as is heterozygosity for HLA alleles (Carrington et al 1999; Kaslow et al 1996; Migueles et al 2000) whereas the effect of a single amino acid change of a particular haplotype of HLA-B35 (Gao et al 2001) and HLA-B22 give less favourable outcomes (Dorak et al 2003).

1.3.2 The role of immune activation

Generally, immune activation through infection with other pathogens, inflammatory stimuli or even HIV-1 infection itself may augment HIV-1 replication, pathogenesis and transmission (Lawn et al 2000a). This may occur through a number of mechanisms.

Firstly, there may be increased expression of chemokine co-receptors on lymphocytes and macrophages, for example following mycobacterial antigen stimulation (Collins et al 2000; Juffermans et al 2000; Wahl et al 1998) or *Pneumocystis carinii* infection (Shaunak et al 2001). Increased expression of CCR5 on CD4⁺ T-cells may be associated with increased viral load and faster HIV associated disease progression (Reynes et al 2000; Reynes et al 2001) but other work has shown interleukin-2 therapy to increase CCR5 expression on CD4 T-cells without increasing HIV-1 viral load (Weissman et al 2000). The increased expression of CCR5 on CD4⁺ T-cells in those living in sub-Saharan Africa is associated with the presence of markers of immune activation and may be driven by environmental factors rather than genetic ones (Clerici et al 2000). There is also evidence that X4 HIV-1 is under represented among HIV-1 strains from sub-Saharan Africa (Ping et al 1999).

Immune activation may also affect HIV disease progression through effects on HIV-1 LTR transactivation (Roebuck et al 1999). Clinical examples of this effect include interactions between HIV-1 and malaria (Xiao et al 1998) and HIV-1 and tuberculosis where significant HIV-1 replication occurs in macrophages secondary to TNF- α stimulation (Alfano et al 1998; Collins et al 2002). NF- κ B mediated HIV-1 replication may also be directly increased by some pathogens such as the gonococcus (Chen et al 2003) and lipopolysaccharide found on gram negative bacteria, though

there is conflicting evidence for this (Alfano et al 1998; Pomerantz et al 1990). The LTR may be directly stimulated, independently of cytoplasmic factors, by *Mycobacterium tuberculosis* (Zhang et al 1995) and by viruses such as HTLV-1, Hepatitis B (Seto et al 1988), CMV (Ho et al 1990), herpes simplex-1 (Mosca et al 1987) and Human herpes virus type 6 (Garzino-Demo et al, 1996).

Immune activation may better predict HIV-1 disease progression than HIV-1 viral load (Leng et al 2001). Apoptosis of HIV-1 infected and uninfected cells via TNF- α mediated pathways may occur though there is conflicting evidence for this (Gougeon et al 1996; Meyaard et al 1994).

All these findings are corroborated by clinical studies demonstrating increased viral loads and greater disease progression associated with mycobacterial disease (Denis et al 1994; Goletti et al 1996; Nakata et al 1997), PCP (Israel-Biet et al 1993) and other infections (Bush et al 1996; Donovan et al 1996; Hoffman et al 1999; Mole et al 1997; Osmond et al 1999; Shaunak et al 2001; Sulkowski et al 1998; Toossi et al 2001). Usually, treatment of these infections results in resolution of immune activation and decrease in viral load. However this is not always the case as in one study on TB where markers of immune activation and viral load remained elevated (Lawn et al 1999).

The genital tract, HIV-1 shedding in semen and sexually transmitted infections

To understand the correlates of HIV-1 shedding in semen requires knowledge of the anatomy and physiology of the male genital tract.

1.4 Anatomy of the male genital tract

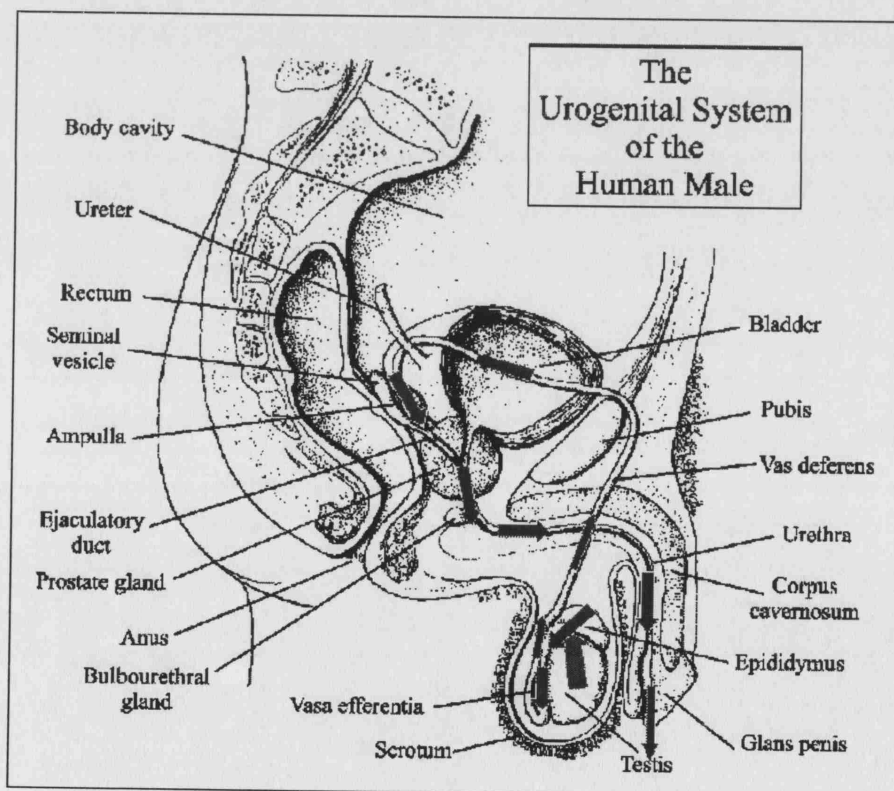
The male genital tract can be thought to begin with the testes. Each of these continues on to an epididymis, then a vas deferens (which passes the seminal vesicles), through the prostate gland by way of the ejaculatory duct, into the prostatic urethra, and finally through the penis in the penile urethra (see Figure 1.3). The testes, gland-like structures, consist of multiple seminiferous tubules within relatively sparse interstitial tissue. The interstitial tissue contains clusters of endocrine (Leydig) cells that secrete testosterone and the seminiferous tubules are lined by a simple columnar epithelium of Sertoli cells among which are interspersed germ cells. Meiotic cell divisions of germ cells result in spermatogonia, which mature, through primary and secondary spermatocytes and spermatids, into spermatozoa. All of the seminiferous tubules converge onto a network of interconnecting tubules, the rete testis, which in turn lead through numerous small efferent ductules to the epididymis. The epididymis consists of the long (4 to 5 meters), convoluted epididymal duct, lined by a two-layered pseudo-stratified columnar epithelium surrounded by smooth muscle. After maturation in the epididymis, sperm are carried to the ejaculatory duct by the relatively straight vas deferens (also lined by two-layered stratified columnar epithelium with a thick, three-layered muscular wall). The vas deferens ends at its confluence with the duct of the seminal vesicle.

The seminal vesicle is a small gland in the shape of a coiled tube with a muscular wall and its highly folded mucosa is lined by a columnar epithelium, similar to that of the vas deferens, producing most of the fluid that makes up human ejaculate. The duct of the seminal vesicle empties into the ejaculatory duct. The ejaculatory duct passes through the prostate, beginning at the confluence of the vas deferens with the

duct of the seminal vesicle and continuing to the prostatic urethra. The prostate, a complex of tubular glands embedded in a thick fibromuscular stroma, surrounds the urethra just below the bladder. Its secretions contribute to seminal fluid. The bulbourethral glands are two pea-sized, lobulated bodies, below the prostate and behind and lateral to the urethra. The excretory duct of each gland, nearly 2.5 cm. long, opens by a minute orifice on the floor of the urethra. Each gland is made up of several lobules, held together by a fibrous coat. The bulbo-urethral glands produce the pre-ejaculatory fluid, and an alkaline fluid contribution to the semen that may serve to neutralize the acidic environment of the urethra, thereby protecting sperm.

Figure 1.3 The Male genital tract

(from <http://www.erin.utoronto.ca/~w3bio380/Lectsked/Lect05/Sperm1.htm> last accessed 05/05/04)



The arrows demonstrate the course of spermatozoa generated in the testes through the genital tract.

In men the urethra extends from the bladder through the prostate and thence out through the penis. Both bladder and prostatic urethra have a muscular wall lined with a transitional epithelium (urothelium), a stratified epithelium in which both cell shape and number of layers can change markedly during the normal process of distension. The penile urethra and bulbo-membranous urethra is lined by simple or pseudo-stratified columnar epithelium and contains in its walls numerous mucus glands (known as Littre's glands) that also produce pre-ejaculatory fluid during sexual arousal. As well as transmitting the urethra, the penis contains erectile tissue, arteries, shunts, and vascular sinusoids that permit erection.

1.4.1 The foreskin and male circumcision

Important external features include the shaft of the penis, the glans and the foreskin (prepuce). In the uncircumcised adult male the glans penis and the preputial under-surface are contiguous and lined by squamous stratified epithelium, a few layers thick. Both animal studies (Miller et al 1994; Miller 1998) and human studies (Hussain et al 1995) have shown this under surface is laden with Langerhans' and mononuclear cells. These cells carry both CD4 receptors and the B-chemokine co-receptor CCR5 on their surfaces and thus are not only targets for HIV infection but may also shed it. More recently the role of mucosal dendritic cells in enhancing infectability of mucosal surfaces by capturing HIV through binding with C-type lectin receptors such as DC-SIGN has been recognised (Pope et al 2003). DC-SIGN expressed on macrophages and dendritic cells has also been demonstrated in human foreskin (Soilleux et al 2004).

A widely accepted model of sexual transmission of HIV in men is based on the genital tract infection of rhesus macaques with simian immunodeficiency virus

(SIV) (Miller 1998; Spira et al 1996). When SIV is inoculated into the penile urethra or onto the foreskin, the virus targets Langerhans' cells located in the mucosa (Miller 1998). Importantly, the average male prepuce has an area of approximately 15 square inches when folded out and this may represent over 50% of the skin surface of the uncircumcised penis (Taylor et al 1996). It is therefore likely that the absence of foreskin reduces the individual risk of acquisition and possibly transmission of HIV for the individual. However whether circumcision in adulthood is an acceptable and effective intervention is unclear and is beyond the remit of this thesis.

1.5 HIV shedding in the male genital tract

1.5.1 Semen and the source of HIV-1 in semen

Normal human ejaculate is a mildly alkaline fluid (pH 7.2 –7.6) with an average volume of 2.5-5ml. The testes and epididymis contribute 10% of semen volume, the prostate 20 to 30% and the seminal vesicles up to 50 to 70% (Malmborg 1978; Pichini et al 1994). The remainder of semen is made of secretions from the urethra and bulbo-urethral glands. These glands are rich in lymphoid tissue (Pudney and Anderson 1995) and can be infected by sexually transmitted infections (STI), for example chlamydia and gonorrhoea.

Semen is dynamically constituted just prior to ejaculation with contributions from different sources. Relative variability in these contributions will result in marked differences in semen composition between individuals and for different ejaculates within individuals. During the first phase of ejaculation, mainly testicular and prostatic fluids are discharged, followed in the later phase by mainly vesicular fluid. Fertility clinics have used this knowledge to develop the split ejaculate technique (where different phases of ejaculate are collected separately) to get concentrated

spermatozoa in first phase ejaculates for artificial insemination. The split ejaculate technique has also been used to relate drug concentrations in different phases of the ejaculate to different parts of the genital tract (van Praag et al 2001a).

The majority of cellular material found in semen consists of mature motile and immotile spermatozoa. Of the remaining cells found in semen, one third consists of immature germ cells, made up of spermatids, spermatocytes and cytoplasmic bodies. The remainder consist of leukocytes and epithelial cells. Polymorphs make up around 90% of seminal leukocytes, lymphocytes 10% and macrophages less than 1% (Coombs et al 2003). Seminal mononuclear cells have been shown to originate from the prostate, seminiferous tubules, the interstitium of the testes and the epididymal epithelium and stroma (Pudney et al 1991). Pre-ejaculatory fluid, secreted by the bulbourethral glands and the glands of Littre, is exposed to sites potentially vulnerable to HIV infection throughout the period of sexual intercourse including sexual arousal and may be important in transmission of HIV. Polymorphs, macrophages, CD4⁺ and CD8⁺ T-lymphocytes have been described in pre-ejaculatory fluid, as has HIV-1 proviral DNA (Pudney et al 1992). Urethral secretions also contribute substantial numbers of leukocytes to semen (Pudney et al 1995).

Studies in vasectomised patients confirm that up to 95% of mononuclear cells and leukocytes are produced in the testes (Anderson et al 1991). However, neither cell free or cell associated levels of HIV-1 in semen are affected up to 3 months after vasectomy according to a study among heterosexual, Thai, injecting drug using men, (Krieger et al 1998). Furthermore, evidence from phylogenetic studies by two independent research groups suggests cell free virus in semen does not arise from seminal leukocytes (Gupta et al 2000; Paranjpe et al 2002; Zhu et al 1996). Further evidence from one of these groups suggests that distinct HIV-1 virion populations are

found in testicular and prostatic tissues (Paranjpe et al 2002). In contrast, other work suggests concordance between seminal leukocyte and seminal plasma HIV-1 strains (Curran et al 2002). Research on macaques has demonstrated SIV at all levels of the genital tract (Miller et al 1994) including virus-containing macrophages and Langerhans' cells found in the epididymis and foreskin respectively. Thus, in humans the major source of HIV-1 virus and provirus in seminal plasma is likely to be distal to the vas deferens, and though the exact sources remain unclear, the prostate, seminal vesicles and urethra are probably major contributors to seminal plasma HIV-1 RNA. Importantly the source of HIV may potentially change during genital tract inflammation such as orchitis (Pudney et al 1991) though in one study, the phylogenetic differences between blood and semen during urethritis did not increase (Ping et al 2000).

1.5.2 The genital compartment

The discordance in blood and semen plasma viral loads in some patients, the variable penetration of antiretroviral drugs into semen and the discrepancies of HIV-1 drug resistant mutations in blood and semen (see below) has led many to propose that the genital tract is a separate compartment in which HIV-1 may replicate at least partially independent of replication in the systemic circulation.

Early studies assessing the dynamics of viral replication in plasma using potent combination antiretroviral therapy to (theoretically) completely and abruptly stop viral replication have suggested that the half-life of free HIV-1 virions in plasma is approximately 6 hours (Ho et al 1995a; Perelson et al 1996; Perelson et al 1997) with up to 10^9 - 10^{10} virions produced daily. The half-life of productively infected cells in these studies was estimated to be approximately 1½ days. More recent studies have

suggested that free virion and infected cell clearance and production from blood plasma may be more rapid (Ramratnam et al 1999; Zhang et al 1999a). Very little data is available on the turn-over rates of virus in the genital tract. In a small study comparing decay of HIV-1 RNA viral load in semen and blood plasma following initiation of combination antiretroviral therapy, decay characteristics in semen were similar to those in plasma, though clearance rates were longer compared to previous studies (Taylor et al 2001b). In a study in women starting zidovudine monotherapy, HIV-1 RNA was cleared considerably more rapidly from the genital tract compared to plasma (Mbori-Ngacha et al 2003). However this finding may have been the result of the higher levels of zidovudine found in the genital tract compared with plasma (see section 1.5.7). Thus it is still unclear whether the higher viral loads observed in the semen of some men is due to greater replication or poorer clearance of virus from the genital tract.

1.5.3 HIV-1 in germ cells

Considerable scientific debate still exists as to whether germ cells are infected with HIV-1. Various studies using different techniques, including immunohistochemistry and electron microscopy (Baccetti et al 1991; Bagasra et al 1988), in-situ hybridisation (Nuovo et al 1994) and in-situ PCR (Baccetti et al 1994), have provided evidence in favour of germ cell infection (Bagasra et al 1994). Studies using in-situ PCR have detected HIV-1 proviral DNA in the spermatozoa of 33% of semen samples of HIV-1 infected men (Bagasra et al 1994) and appeared to demonstrate presence of HIV-1 nucleic acid and antigen within normal spermatozoa (Baccetti et al 1994). Another group using in-situ hybridisation detected proviral DNA in the spermatogonial and spermatocyte populations within the interstitium of

the testes (Baccetti et al 1994; Nuovo et al 1994; Shevchuk et al 1998). However attempts by independent groups to replicate the findings of these studies using a variety of techniques including in-situ PCR, in-situ hybridisation and electron microscopy have consistently failed to demonstrate germ cell infection by HIV-1 (Pudney et al 1999). Furthermore, studies using immunomagnetic beads to separate seminal cells suggest that T-lymphocytes and macrophages, but not spermatozoa, are infected with HIV (Pudney et al 1999; Quayle et al 1997; Quayle et al 1998). More recently, however, HIV-1 RNA and DNA were found in selected spermatozoa of 6/34 and 1/34 men not on antiretroviral treatment respectively, undergoing sperm washing for the purposes of assisted reproduction (Leruez-Ville et al 2002a). Sperm may also potentiate transmission of HIV by initially binding HIV-1 gp120 to a galactoglycerolipid, found on the mid-portion of sperm tails (Brogi et al 1996), and then by binding to HLA-DR, found on activated immune cells (Scofield 1998).

Despite these controversies, it is unlikely that HIV-1 infected spermatozoa are an important source for transmitting HIV-1 as no HIV transmissions have occurred in two large series of women undergoing artificial insemination (Marina et al 1998; Semprini et al 1997) nor in a study of in-vitro fertilisation with intra-cytoplasmic injection with appropriately washed spermatozoa from HIV-1 infected semen (Pena et al 2003).

1.5.4 HIV-1 viral loads in semen

Among those not on antiretroviral therapy with chronic HIV infection studies have shown in general a good correlation between HIV-1 RNA load in seminal and blood plasma (Ball et al 1999a; Dulioust et al 1998; Merigan 1998; Tachet et al 1999; Vernazza et al 1997a) though some studies have not shown this correlation (Coombs

et al 1998; Fiscus et al 1998; Kalichman et al 2001; Liuzzi et al 1996; Tachet et al 1999; Vernazza et al 1997b). Semen plasma viral loads (SPVL) are generally lower than blood plasma viral loads (BPVL) by between 3 to 10-fold but most comparisons report a minority of patients where seminal values are higher, perhaps in up to 30% of patients (Ball et al 1999a; Coombs et al 1998; Dulioust et al 1998; Leruez-Ville et al 2002a; Liuzzi et al 1996). This relationship appears to be true, even for the very high viral loads found in those with acute HIV-1 infection, though absolute values in semen are variable and begin to decline after the onset of symptoms (Dyer et al 1997; Pilcher et al 2001).

Correlation of viral titres between blood and semen may be dependent on the pattern of shedding of HIV-1 in semen. HIV-1 may be shed in semen intermittently over time in some patients and continuously or not at all in others (Gupta et al 2000; Krieger et al 1995). In those who shed continuously there appears to be a good correlation between semen and blood plasma viral loads, with viral populations in semen plasma phylogenetically similar to those in blood plasma. These findings stand in contrast to those who shed HIV-1 intermittently (Gupta et al 2000) suggesting that in intermittent shedders, HIV-1 in semen is generated within the genital tract.

Recently, it has been suggested that correlation between semen and blood plasma viral HIV-1 RNA loads is dependent on stage of HIV disease with patients with a CD4⁺ T-cell count less than 200 x 10⁶/ml having no correlation, those with CD4⁺ T-cell counts between 200 and 500 having a good correlation and those with a CD4⁺ T-cell count of greater than 500 having the strongest correlation (Pinto-Neto et al 2002). In this study, SPVL was similar to BPVL in those with CD4⁺ T cell counts greater than 500 but lower than BPVL by between 0.6 and 0.9 log in those with CD4⁺ T cell counts between 500 and 200 and less than 200 respectively. In contrast to

previous work (Coombs et al 1998), no correlation between SPVL and CD4⁺ T cell count was demonstrated, suggesting that those with earlier HIV disease shed similar amounts of HIV in semen than those with late disease.

HIV-1 proviral DNA is found in 100% of blood samples and approximately 50% of non-spermatozoal fractions (Ball et al 1999a; Dulioust et al 1998; Tachet et al 1999; Xu et al 1997). DNA levels are generally higher in blood compared with semen and may be correlated with it (Xu et al 1997). Both cell-free and cell-associated variants in semen have been shown to be transmissible (Zhu et al 1996).

1.5.5 HIV-1 loads in semen predict infectiousness

Epidemiological data from numerous studies demonstrate an association between transmission of HIV infection and blood plasma viral loads. These imply, though do not prove, that there is a similar association between genital tract viral loads and sexual transmission. A strong relationship between levels of HIV-1 RNA in seminal plasma and the ease of co-culture of HIV-1 from peripheral blood mononuclear cells (PBMC) has also been demonstrated, with the highest likelihood of co-culture in those with high seminal plasma viral loads (Coombs et al 1998; Vernazza et al 1997b). Another study has demonstrated that the ability to culture HIV-1 from seminal cells is related to HIV-1 RNA levels in seminal plasma and HIV-1 DNA levels in non-spermatozoal seminal cells (Tachet et al 1999). Levels of semen DNA have also been associated with the ability to culture virus in PBMCs (Xu et al 1997). Assuming the ability to culture virus is associated with infectiousness, these data imply that SPVLs are associated with infectiousness. However there is evidence that other factors assumed to be important for transmission, for example macrophage tropism of HIV, bears no relationship with seminal plasma viral load (Coombs et al

1998) and whether increases in SPVLs secondary to inflammation in the genital tract increases infectiousness has not been demonstrated despite clear epidemiological associations between genital tract infection and transmission of HIV (see section 1.7).

1.5.6 Effects of antiretroviral therapy on semen HIV-1 RNA and DNA loads

In those on antiretroviral therapy, suppression of BPVL is largely associated with suppression of SPVL (Barroso et al 2000; Gupta et al 1997; Leruez-Ville et al 2002b; Liuzzi et al 1999; Vernazza et al 1997c; Vernazza et al 2000) but low RNA levels in seminal plasma have been found in a minority of patients with undetectable BPVL on potent therapy (Leruez-Ville et al 2002b; Vernazza et al 2000). Recently, of 56 patients on suppressive CART for 18 months, with HIV-1 RNA viral loads in blood less than 20 copies/ml, seminal viral loads were found to be detectable ranging from 84 to 3224 copies/ml (Leruez-Ville et al 2002a), suggesting that significant viral replication occurs independent of the systemic circulation. Antiretroviral therapy also reduces blood and semen HIV-1 proviral DNA levels (Vernazza et al 2000). Replication competent provirus, with evidence of ongoing viral replication, has been found in both the blood and semen of patients on suppressive combination antiretroviral therapy when levels of HIV-1 RNA in these respective compartments have been undetectable (Andreoni et al 2000; Zhang et al 1998; Zhang et al 1999b). In addition, though the presence and quantity of HIV-1 proviral DNA in semen cells is correlated with both SPVL and BPVL, seminal HIV-1 DNA has been found in patients with undetectable SPVL and BPVL (Ball et al 1999a; Liuzzi et al 1999; Tachet et al 1999; Zhang et al 1998). It is important to note here that although a study, using quantification of 2-LTR DNA circles as a marker of recent reverse transcription failed to detect evidence of on-going viral replication in semen cells (Nunnari et al

2002), this work has been called into question by the refutation of this method (Brussel 2003; Bushman 2003).

1.5.7 Concentration of antiretrovirals in semen

The ability of antiretrovirals to suppress genital tract viral load and their potential to select drug resistant virus will depend mainly on the concentrations they achieve in this compartment. As the male genital tract is not easily accessible numerous investigators have measured seminal plasma concentrations of antiretrovirals and attempted to relate them to viral inhibitory concentrations in in-vitro cell cultures as conventionally done for blood plasma concentrations. However, because of the dynamic way in which semen is formed and the variability of its composition, semen concentrations of drugs may not necessarily accurately reflect true concentrations of drug in different parts of the genital tract. Additionally, the frequency of ejaculation may potentially alter composition of seminal fluid, drug concentrations and HIV-1 viral load in semen. Conventionally, total drug concentrations in plasma are measured, (including free drug and that which is protein bound). With significant protein binding, levels of free drug and its availability for antiviral activity may be significantly affected. Importantly, human semen is a highly proteinaceous fluid. Protein binding of any drug is difficult to measure and as yet no studies have investigated its effects on free drug concentrations in semen. Nevertheless, these investigators have thrown considerable light onto genital tract penetration of antiretroviral drugs.

Generally nucleoside analogue reverse transcriptase inhibitors achieve high concentrations in semen. Zidovudine and lamivudine may be particularly concentrated in seminal plasma at levels considerably higher than in blood plasma (Anderson et al

2000; Henry et al 1988; Pereira et al 1999; Pereira et al 2002). Stavudine and abacavir are found at levels similar or slightly below those found in blood (Reijers M et al 2000; Taylor et al 2000; van Praag et al 2001b). At the time of writing, no published data on semen levels of tenofovir, didanosine, ddC and emtricitabine exist.

Of the non-nucleoside reverse transcriptase inhibitors, nevirapine has been found at levels in whole semen comparable to those found in blood (Taylor et al 2000) and in a study using the split ejaculate technique, at therapeutic levels in prostatic and testicular secretions (van Praag et al 2001a). In a short prospective study of 19 HIV-1 infected men starting an antiretroviral regimen containing efavirenz, semen plasma concentrations of efavirenz were approximately 10% of those in blood and changed very little over the dosing interval. However, blood and semen plasma viral loads were maximally suppressed on the drug regimens (Taylor et al 2001c). In a similar study in 9 efavirenz naïve and 12 efavirenz experienced patients the average efavirenz exposure in semen over the dosing interval was only 3.4 % of that in blood but in all patients semen plasma concentrations remained above the wild-type IC_{90} for HIV-1 (Reddy et al 2002).

Recent studies have suggested that there is poor penetration of protease inhibitors into the genital tract. An exception to this is indinavir (Taylor et al 2001a; van Praag et al 2001a) where levels equivalent to those in blood have been found. Ritonavir, saquinavir, nelfinavir and lopinavir have been found at low concentrations in seminal plasma (Reijers M et al 2000; Sankatsing et al 2003; Taylor et al 1999; Taylor et al 2001a). Lopinavir levels remain low even, in the presence of ritonavir, and are unrelated to the time since ingestion of the drug (Lafeuillade et al 2003). In a study on amprenavir levels in semen in 31 men, median drug concentrations in semen were approximately 14% of those in plasma. In a sub-group of patients who received

amprenavir monotherapy, the suppressive effect on HIV-1 viral load in semen was related to semen plasma amprenavir concentrations (Pereira et al 2002). In another study, the majority of amprenavir concentrations measured in semen were greater than the plasma protein-corrected 50% inhibitory concentration for wild-type human immunodeficiency virus type 1 (Chaudry et al 2002).

1.5.8 Drug-resistant variants in the genital tract

Though combination therapies have been highly successful in reducing morbidity from HIV infection by suppressing virus and improving immunity, the development of drug resistance has led to increasing therapeutic failure of these regimes. Furthermore, there is evidence that the sexual transmission of drug resistant and multi-drug/class resistant variants of HIV-1 may be increasing (Boden et al 1999; Brodine et al 1999; Erice et al 1993; Magiorkinis et al 1999; Yerly et al 1999) (Hasse et al 2003; Little et al 2002; Simon et al 2002; UK Collaborative Group on Monitoring the Transmission of HIV Drug Resistance. 2001). Some drug resistance mutations may decrease the replicative capacity of HIV-1 in patients on therapy (Nicastrì et al 2003) and make virus less transmissible than wild-type (Leigh Brown et al 2003) but other mutations may have the converse effect (Chan et al 2003; Simon et al 2003). Drug resistant virus is capable of being transmitted through a chain of at least 3 individuals (Taylor et al 2003a) and furthermore as HIV-1 is known to super-infect patients with chronic HIV infection (Altfeld et al 2002; Jost et al 2002; Koelsch et al 2003) sexual transmission of drug resistant virus has the capacity to undermine current therapies as well as limiting future ones. Superinfection, however is likely to be a rare event (Gonzales et al 2003). The development and evolution of drug resistant strains in the genital tract is thus important to study.

A number of studies have demonstrated that drug and multi-drug resistant mutations in patients on combination therapy evolve in blood and semen apparently either concurrently in both compartments or independently, with frequent evolution of mutations in one compartment and not the other. This has shown to be true for both cell associated provirus (Byrn et al 1997; Byrn et al 1998; Eyre et al 2000; Kiessling et al 1998; Kroodsma et al 1994) and cell free virions found in plasma (Eron et al 1998; Lafeuillade et al 2003; Merigan 1998). In patients where concordance of drug resistance mutations is found it is unclear whether resistant virus has developed in one compartment and seeded in to the other or developed independently in both. Evidence exists that loss of virological control in the genital tract, due to poor penetration of antiretrovirals, may precede that in the systemic circulation, but evidence of sequential development of drug resistance is lacking (Ball et al 1999b).

1.6 Factors associated with increased risk of HIV transmission

Various factors determine the infectiousness of HIV-1 in semen, including the viral load, the chemokine receptor status of the recipient and donor, perhaps the viral subtype and whether the virus is cell associated or cell free.

1.6.1 The role of viral load

Probably the most important determinant of sexual infectiousness of an individual is the quantity of virus in genital secretions. Evidence to support this statement is inferred from epidemiological studies showing an association between blood plasma viral loads and transmission and biological data correlating genital viral loads with infectiousness in-vitro (see below). Heterosexual transmission of HIV-1 between discordant partners is strongly associated with blood plasma viral load both

in developed and developing world settings (Fideli et al 2001; Hisada et al 2000; Lee et al 1996; Operskalski et al 1997; Pedraza et al 1999; Quinn et al 2000). Further evidence of the importance of viral loads in transmission comes from studies on children born to mothers with HIV infection (Sperling et al 1996), however at the time of writing there are no published data on the association of viral loads and transmission risk among homosexual men.

In a large study from Rakai, Uganda, of 415 initially discordant partners, viral loads of transmitting couples were considerably higher than non-transmitting couples and no transmissions occurred when the infected partner had a viral load of less than 1500 copies/ml (Quinn et al 2000). Though some small studies have suggested that HIV transmission rates are higher from men to women than vice versa (O'Brien et al 1994; Padian et al 1997; Saracco et al 1993) larger studies now confirm that risks of heterosexual transmission for both sexes are similar (Allen et al 1992) even when stratified by viral load in the index case (Fideli et al 2001; Quinn et al 2000; Serwadda et al 1995). Difficulties arise when extending the findings of these data to patients who are not in HIV sero-discordant steady relationships. This is because HIV negative individuals in sero-discordant relationships may represent a group that is, by its very nature, relatively "resistant" to HIV infection and thus estimates of transmission risk may be underestimates. Evidence exists that patients constantly exposed to but uninfected by HIV may have relative protective immunity against HIV-1 (Rowland-Jones et al 1999). Furthermore, blood viral load may be a poor predictor of genital viral load in those who have regular casual as opposed to monogamous relationships because of differences in rates of acquiring sexually transmitted infections. This caution about using blood plasma viral loads to infer transmission risk is emphasised in a large study, which demonstrated a weaker association between blood plasma viral

loads and transmission from HIV infected men compared to transmission from HIV infected women (Fideli et al 2001).

1.6.2 The role of chemokine co-receptors

The importance of the role of CCR5 in sexual transmission of HIV-1 is emphasised by the relative resistance to sexual and vertical transmission of HIV-1 in those individuals homozygous for a defective CCR5 allele, delta 32/delta32 (Paxton et al 1998a; Philpott et al 1999; Wu et al 1997). The delta-32 allele is relatively common among Caucasians with a frequency of 0.08 but absent in Asians and Africans.

Although both R5 and X4 viruses are transmissible, R5 virus tends to be more readily transmitted and there is evidence that minority populations of virus found in semen are selected for transmission (Zhu et al 1996). However both R5 and X4 viruses are present in genital secretions of HIV-1 infected patients implying that selection of R5 virus during transmission may also depend on factors in the recipient (Delwart et al 1998; Sutthent et al 2001).

The female and perhaps the male genital tract contain larger concentrations of T-cells carrying CCR5 compared with the peripheral circulation and the concentration of CCR5 on individual cells may also be moderately increased (Hladik et al 1999). Evidence exists that increasing the concentration of co-receptors on individual cells affects susceptibility of T-cells and macrophages to infection with HIV-1 (Paxton et al 1998b; Wu et al 1997). Other evidence suggests that the expression of the CD4 receptor on cells is more important, such that even low expression of CCR5 results in efficient infection of susceptible cells (Pesenti et al 1999).

The number of CCR5-expressing cells is increased at sites of genital inflammation as is the concentration of CCR5 expressed on those cells (Hladik et al 1999; Qin et al 1998; Rottman et al 1997). Inflammation also increases the numbers of CD4⁺ cells on the endocervix (Levine et al 1998).

In-vitro work supports the role of the US28 gene product of *Human cytomegalovirus* in facilitating HIV-1 transmission by acting as a co-receptor for HIV-1 on otherwise resistant CD4⁺ T cells (Pleskoff et al 1998). Co-infection with another herpes virus, *Human herpes virus 6*, may increase susceptibility to HIV-1 infection by increasing numbers of CD4 receptors on CD8⁺ T-cells (Lusso et al 1995).

1.6.3 The role of HIV-1 subtype

It is unclear whether HIV-1 subtype affects the sexual transmission of HIV-1. Evidence that HIV-1 CRF_AE (formerly subtype E) grows more efficiently than subtype B in Langerhans' cells has not been confirmed in subsequent research (Dittmar et al 1997; Soto-Ramirez et al 1996). Semen viral loads have been found to be similar in those with HIV-1 subtypes E and B (Coombs et al 1999). Although viral loads in the genital tract and blood may be higher in sub-Saharan Africa compared with the west (Dyer et al 1998b) these higher loads may be driven by environmental factors (Clerici et al 2000). However viral factors associated with subtype may play a part in infectivity. For example, the LTR of subtype C has 3 or more binding sites for NFκB compared with only 2 in subtype B (Montano et al 2000) arguably making it more sensitive to the effect of this transcription factor. Finally, as discussed earlier, X4 virus (thought to be less transmissible than R5 virus) may be under represented in African sub-types (Ping et al 1999).

1.6.4 Cell free versus cell associated virus

The importance to HIV-1 transmission of whether the virus is cell-associated or cell-free is unknown. In a study of five acute HIV-1 seroconverters and their transmitting partners, the transmitted virus was found in the seminal plasma but not the seminal cells of one index case and, in three other index cases, in the seminal cells only. The data suggest that both cell-free and cell-associated virus may be transmissible (Zhu et al 1996). In-vitro work has demonstrated that HIV-1 infected mononuclear cells may release virions near epithelial membranes with subsequent transcytosis across the epithelial barrier and infection of subepithelial mononuclear cells (Bomsel 1997; Bourinbaier et al 1991; Tan et al 1993). Other possible mechanisms of cell associated transmission of HIV-1 include infection of epithelial cells through fusion of microvilli of infected mononuclear cells with epithelia (Bourinbaier et al 1991) or transmigration of macrophages or lymphocytes across epithelia (Zacharopoulos et al 1997).

1.7 The role of sexually transmitted infections

A number of epidemiological studies provide evidence that STIs increase the probability of HIV-1 transmission by increasing susceptibility and infectiousness (Fleming et al 1999; Rottingen et al 2001). Numerous biological mechanisms may explain these effects including recruitment of activated target cells (CD4⁺ T-lymphocytes and macrophages) for HIV to inflamed genital tissue (including genital ulcers), expression of pro-inflammatory cytokines in genital mucosa and increased genital shedding of HIV-1 in infected patients.

Several STI-intervention trials have highlighted the influence of STIs on HIV-1 transmission. In Mwanza, Tanzania, syndromic management of bacterial STIs

reduced HIV-1 incidence by 40% (and also the prevalence of STIs) (Grosskurth et al 1995). However, two studies from Uganda, the Masaka trial (Kamali et al 2003), based on syndromic bacterial STI management with or without a behavioural intervention, and the Rakai study (Wawer et al 1998), based on periodic mass STI treatment, demonstrated no impact of STI treatment on HIV-1 transmission. Explanations for the lack of an effect in these two trials have included low baseline levels of bacterial STIs, the higher HSV-2 prevalence in Uganda and the more mature HIV epidemic of Uganda compared with Mwanza.

1.7.1 Herpes simplex infection

Epidemiological and biological data support a strong association between genital herpes infection and HIV-1 acquisition and transmission. HSV-2 is the most common cause of genital ulcer disease (GUD) worldwide. Over 60% of GUD in many STI clinics in the US are caused by HSV (Mertz et al 1998) and the proportion of GUD caused by HSV-2 infection may be increasing in many developing countries. In South Africa which had a fast growing HIV epidemic during the 1990s the proportion of genital ulcers attributable to HSV-2 infection grew from 12% to 21%, a result not due to increased sensitivity of testing (Lai et al 2003). Infection with HSV-2 is also highly prevalent with 22% of sexually active adults in the US (Fleming et al 1997), and over 50% in some populations in sub-Saharan Africa infected (Weiss et al 2001). These prevalence rates are much higher among those with HIV-1 infection. In one study among homosexual men in a primary care setting in the developed world the prevalences of HSV-2 antibodies were 60% and 20% in men with and without HIV-1 infection respectively (Russell et al 2001). Prior infection with HSV-2 considerably increases the risk of HIV-1 acquisition. In one meta-analysis of studies estimating the risk of HIV-1 infection associated with being seropositive for HSV-2, population

attributable risk was 19% in populations with 22% HSV-2 prevalence, increasing to 47% in populations with 80% HSV-2 prevalence, a level existing in many parts of sub-Saharan Africa (Wald et al 2002). In Uganda, HSV-2 infection among HIV-1 negative partners increased their risk of acquiring HIV-1 by approximately 70% whereas urethritis and laboratory-confirmed STIs did not increase the risk of HIV-1 acquisition (Serwadda et al 2003). Among men in Tanzania, being serologically positive for HSV-2 or becoming infected with HSV-2 was associated with HIV-1 sero-conversion over a 2 year follow up (Mar Pujades et al 2002).

Considerable biological evidence exists to support the influence of HSV-2 infection on the sexual transmission of HIV-1. Outbreaks of herpes simplex infection increase blood plasma HIV-1 viral load and anti-HSV treatment may reduce HIV-1 load (Mole et al 1997). This may explain the increased shedding of HIV-1 from genital ulcers during recurrent herpetic episodes (Gadkari et al 1998; Schacker et al 1998a). In a study from South Africa, syndromic treatment of genital ulcer disease resulted in reduction of HIV-1 recovered from lesions though it was not clearly stated what the causes of genital ulcer disease were (Ballard RC et al 1999). In women HIV-1 may be increasingly shed during subclinical HSV-2 reactivations (McClelland et al 2002) and reactivation of HSV-2 is common in many immunocompetent HIV-infected people further increasing the risk of HIV transmission (Schacker et al 1998b). Formation of *Human herpes virus 1*/HIV-1 pseudotypes, potentially expanding the tropism of HIV-1 to epithelial cells has been demonstrated in vitro but the findings require confirmation (Calistri et al 1999; Heng et al 1994).

1.7.2 Urethritis

Urethritis, a syndrome of urethral symptoms and signs such as urethral discharge, irritation and dysuria may be caused by a number of sexually transmitted infections. The major pathogens involved include *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Mycoplasma genitalium* and *Ureaplasma urealyticum* though many other organisms have been implicated (Holmes KK et al 1999). The diagnosis of gonococcal urethritis is made relatively simply by the observation of gram-negative intracellular diplococci on a urethral smear or positive cultures from a urethral swab. The diagnosis of non-gonococcal urethritis (NGU) in symptomatic males or in the presence of discharge is generally straightforward but is more difficult in men without symptoms or signs. In these cases, clinicians rely on the presence of excess polymorphs in the anterior urethra to establish the diagnosis.

UK guidelines for the management of NGU, state that diagnosis must be confirmed either by demonstrating greater than 4 polymorphonuclear leucocytes per high power field (p/hpf) or greater than 9 p/hpf on a gram-stained smear from the anterior urethra or from the sediment of a sample of first-pass urine respectively (Clinical Effectiveness Group (Association of Genitourinary Medicine and the Medical Society for the Study of Venereal Diseases) 1999). The high power field represents a magnification of x1000 using a light microscope (combining a x100 objective lens with a x10 eyepiece). The use of 5 p/hpf as a cut-off between normal and NGU has historically come from research studies at a time when sensitive and specific molecular techniques for detecting pathogens had not been developed. There is some difficulty when attempting to extend the findings of these studies to the

asymptomatic patient (Arya et al 1984; Bowie 1978; Desai et al 1982; Landis et al 1988; Terry et al 1991).

Additionally, diagnosis of NGU by microscopy will vary depending on a number of factors including the time since last passing urine, the method by which the urethral specimen is taken and applied to the slide and the subjectivity and skills of the microscopist examining it.

Infection with *Chlamydia trachomatis* or *Neisseria gonorrhoeae* may cause significant genital inflammation or be asymptomatic. Chlamydia, an obligate intracellular parasite almost exclusively infects squamo-columnar epithelia. The process may be highly immunogenic and hypersensitising. Infection with *N. gonorrhoeae*, an extracellular pathogen, results in inflammation perhaps caused by extracellular toxins proteins or through hypersensitivity. Though an extra cellular pathogen, it is avidly phagocytosed by neutrophils (Holmes KK et al 1999).

Studies have shown that in those not on antiretroviral therapy, SPVL and proviral HIV-1 DNA concentrations in semen and genital secretions may increase considerably during episodes of gonococcal, non-gonococcal and chlamydial urethritis and fall following treatment for the urethritis (Atkins et al 1996; Cohen et al 1997; Eron, Jr. et al 1996; Moss et al 1995; Winter et al 1999). In Africa, median SPVLs were over 100,000 copies/ml higher in cases of gonococcal urethritis (GU) compared to those without STIs (Cohen et al 1997). From the same study, symptomatic NGU with *Trichomonas vaginalis* infection as the sole pathogen was associated with higher SPVLs compared with NGU where no pathogen was isolated. Asymptomatic carriage of *T.vaginalis* in the urethra was not associated with increased shedding in this study though this finding may have been the result of a small sample size (Hobbs et al 1999). Studies from the developed world indicating an effect of

urethritis on HIV-1 RNA and proviral DNA loads in semen have been small (Eron, Jr. et al 1996; Winter et al 1999).

In-vitro studies have given conflicting evidence of the effect of *Chlamydia trachomatis* on HIV-1 replication, some showing enhancing effects (Ho et al 1995b) and others showing inhibitory effects (Bianchi et al 1998). One in-vitro study has demonstrated increased activation of the LTR mediated by *N. gonorrhoeae* (Chen et al 2003). It is possible that gonococcal urethritis is more likely to raise SPVL than non-gonococcal urethritis because of the more pronounced inflammatory effect of the former (Cohen et al 1997).

It is also possible that the effects of STIs on SPVLs, observed in Africa may be greater than those observed in the developed world. HIV-1 infected individuals in Africa have, on average, higher blood plasma viral loads (BPVL) and states of immune activation than those in the developed world (Clerici et al 2000; Dyer et al 1998b) and the effects of inflammatory cytokines on viral replication may be greater on prevalent sub-types in Africa compared to subtype B, found more commonly in Europe and North America (Montano et al 2000). Furthermore, delayed health seeking behaviour of those with STIs in the developing world may allow STIs to have greater impact before treatment is given (Faxelid et al 1998).

The effects of urethritis in patients taking CART have not been studied. It is possible that STI-induced viral replication in the presence of CART, may result in the generation of drug resistant virus in the genital compartment with the potential of onward sexual transmission.

1.7.3 Syphilis

Early syphilis has two symptomatic stages: primary syphilis, characterised by the primary chancre usually with little in the way of systemic features, and secondary syphilis characterised by a mild to severe systemic illness. Early latent syphilis is defined when an asymptomatic patient presents with positive syphilis serology after having had negative serology in the previous two years, or who has become asymptomatic within two years of untreated primary or secondary syphilis. These distinctions are made as *Treponema pallidum* (the cause of syphilis) is known to replicate more actively in the first 2 years following infection, impacting on treatment choices and the likelihood of sexual transmission. Diagnostically, the rapid plasma reagin test (RPR), which measures anti-cardiolipin antibodies, is positive at high dilutions during secondary syphilis (usually a minimum of 1:16) but may be low or negative in primary syphilis. Treponemal-specific tests however, such as the fluorescent treponemal antibody are usually positive at this stage. Successful treatment during primary or secondary syphilis usually results in a decline of the RPR to negative (or at least in a fourfold decline to a stable RPR titre) over the course of three to six months (Clinical Effectiveness Group (Association for Genitourinary Medicine and the Medical Society for the Study of Venereal Diseases) 2002). Constitutional symptoms usually take a matter of days or weeks to resolve following a single injection of Benzathine Penicillin.

The sexual transmission of syphilis and of HIV-1 has been linked epidemiologically (Darrow et al 1987; Ellerbrock et al 1992; Fleming et al 1999; Kuiken et al 1990; Otten, Jr. et al 1994). In the UK, there has been an increase in the incidence of STIs including early syphilis and the latter has affected a substantial

number of homosexual men with HIV-1 infection (Crook et al 2002; Doherty et al 2002).

The effects of HIV infection on the course and presentation of early syphilis has recently been described in a large multicentre study (Rompalo et al 2001) comparing 101 HIV-infected patients to those without HIV infection. This study showed that the numbers of ulcers in patients with primary syphilis or secondary syphilis were increased in HIV-infected patients. During secondary syphilis RPR was higher in HIV-infected patients as compared to HIV negatives (median 1:256 vs 1:128 respectively, $p < 0.05$). HIV-infected patients also failed therapy by titre decline more frequently and may have had more aggressive syphilis. The study was not adequately powered to determine differences in presentation at different levels of CD4⁺ T-lymphocyte count within HIV-infected patients.

Recent work in-vivo has demonstrated that increased expression of CCR5 on CD4⁺ T-lymphocytes is associated with increased HIV-1 viral load and faster HIV-1 disease progression (Reynes et al 2000; Reynes et al 2001). This is corroborated by studies on tuberculosis: in-vitro work has shown that mycobacterial antigens increase chemokine co-receptor density on monocytes (Collins et al 2000; Juffermans et al 2000) and clinically, active tuberculosis is associated with higher viral loads and greater HIV disease progression (Goletti et al 1996; Toossi et al 2001).

T. pallidum and its associated antigens have also been shown to increase chemokine co-receptor density on macrophages (Sellati et al 2000) and in addition, to activate NF κ B in monocytes (Norgard et al 1996). Through this latter mechanism *T. pallidum* was shown to increase HIV-1 gene expression (Theus et al 1998). In-vitro, treponemal antigens activate macrophages to produce tumour necrosis factor- α (TNF- α) (Radolf et al 1991; Radolf et al 1995) and secondary syphilis is associated with

increased production of chemokines such as interleukin-2 (IL-2), IL-12, interferon- γ (IFN- γ) (Van Voorhis et al 1996). Interestingly, these chemokines and others including (TNF- α) can all induce CCR5 expression on lymphocytes (Reynes et al 2000).

Syphilis skin lesions contain both CD8⁺ and CD4⁺ T-cells with perhaps the latter predominating in primary chancres (Engelkens et al 1993; McBroom et al 1999; Tosca et al 1988). In Africa, genito-ulcerative disease has been associated with increased shedding of HIV-1 from the female and male genital tracts (Dyer et al 1998a; Ghys et al 1997; Lawn et al 2000b). The painless nature of the primary chancre may be important in facilitating HIV acquisition and transmission, particularly when considering chancres in concealed sites such as beneath the foreskin, in the rectum or on the cervix.

Collectively these data suggest that during early syphilis there is increased susceptibility to HIV-1 infection. In those already infected with HIV-1 there may be increased virus shedding in genital and oral chancres and in genital secretions resulting in increased transmissibility of HIV-1. Additionally, the evidence suggests that during secondary syphilis blood plasma HIV-1 viral loads may increase. In those on antiretroviral therapy early syphilis may compromise control of HIV replication and potentially increase the likelihood of failure of therapy. In those not on therapy early syphilis may impact on progression of HIV-1 disease.

1.8 Difficulties in research involving donation of semen

This thesis describes a series of studies in which semen needed to be collected from patients with symptomatic sexually transmitted infections. The study of the biology of HIV-1 in semen is important in understanding HIV-1 transmission (Vernazza et al

1999) but undertaking large studies may be perceived to be difficult because of the requirement of semen donation, particularly in those with STIs. Research into the feasibility of semen collection in humans has been done in the context of semen donation for fertility clinics and semen retrieval from young boys with cancer. In the context of fertility clinics, among those given monetary incentives to take part, young age, higher educational status and financial motivation appear to be important factors associated with donation of semen. Men who volunteer to donate semen without monetary incentives are generally older, have settled families and are motivated mainly by a sense of altruism (Baker et al 2000; Cook et al 1995; Coulson et al 2001; Daniels et al 1997).

Studies involving semen donation have been conducted in men with different STIs including HIV infection (Vernazza et al 1999), *Trichomonas vaginalis* (Gopalkrishnan et al 1990), *Chlamydia trachomatis* (Keck et al 1998), *Herpes simplex* (Wald et al 1999) and non-specific urethritis (Witkin et al 1983). Fewer semen studies have attempted to examine the interactions between HIV and symptomatic STIs and, in the developed world, these have tended to be small studies (Atkins et al 1996; Cohen et al 1997; Dyer et al 1998a; Eron, Jr. et al 1996; Winter et al 1999). However, there are no data on the acceptability and feasibility of such research.

CHAPTER 2. LABORATORY METHODS FOR THE STUDY OF HIV-1 IN SEMEN

2.1 Quantifying HIV-1 RNA loads in seminal and blood plasma

In the studies in this thesis where semen and blood plasma HIV-1 RNA loads were assayed, HIV-1 RNA was extracted by a silica gel capture method previously observed to successfully remove inhibitors of PCR (Boom et al 1990). For most samples viral loads were quantified using an in-house, internally calibrated reverse transcribed PCR assay (RT-QPCR, Department of Virology UCL) but in four patients they were was assayed using NASBA (Nuclisens HIV-1 QT, Organon Teknika, Boxtel, Netherlands) as previously described (Dyer et al 1996). We demonstrated a good correlation between these two assays (see section 2.7).

2.2 Collection, separation and storage of blood, semen and urine samples

Patients provided semen samples into sterile containers by masturbating either in their homes or in the clinic. Patients were advised to avoid voiding urine just prior to masturbation, to prevent any urethral discharge being washed out, and using lubricants. Semen samples were processed within two hours of producing the sample and allowed to liquefy by standing at room temperature for 30 minutes and transferred to plastic centrifugation tubes. Blood was collected by venesection into EDTA vacutainer tubes usually within minutes of semen collection (but never more than two hours after semen production). Similarly, a recorded volume of first void urine was collected in some patients prior to masturbation.

Blood, semen and urine plasma were separated from cells by centrifugation of samples at 3000g for 10 minutes and then 0.5ml aliquots placed in sarstedt tubes.

Semen cells were re-suspended in 0.5mls of phosphate buffered saline and the buffy coat layer of the blood cell residue aspirated into sarstedt tubes for proviral DNA analysis. All samples were then stored at -70°C.

2.3 Principle of the in-house RT-QPCR assay

2.3.1 RNA extraction

The method involves the extraction and purification of viral RNA from plasma using adsorption and elution from silica gel particles (Boom et al 1990). HIV-1 RNA is isolated from plasma in a buffer containing a detergent (Triton X-100) to lyse the virus particles and guanidine thiocyanate (GuCSN) to prevent RNAase degradation of the released RNA. Silica gel particles, which bind HIV-1 RNA in the presence of GuCSN, are added, washed and finally RNA eluted into nuclease-free water. This method of extraction has previously been shown to successfully remove inhibitors of PCR found in semen (Dyer et al 1996).

2.3.2 RT-QPCR

PCR is an in-vitro cyclical reaction in which defined DNA sequences are replicated resulting in a doubling of the number of copies of the sequence in each cycle, hence generating a very large ($>10^{12}$) number of copies after multiple (35+) cycles. The process involves two oligonucleotide primers (roughly 15 to 30 bases long) that are complementary to nucleic acid sequences that flank the target sequence to be amplified. To amplify RNA, the RNA must be converted to complementary DNA (cDNA) with the enzyme reverse transcriptase prior to amplification. In this method, the reaction is performed with thermo-stable recombinant enzyme *Thermus thermophilus* DNA polymerase (r*Tth* pol) which has both reverse transcriptase (RT)

and DNA polymerase activity in the presence of manganese and the appropriate buffer conditions allowing both reverse transcription and PCR amplification to occur in a single tube reaction. The reaction mixture is heated to allow the downstream primer to anneal specifically to the target RNA and in the presence of excess deoxynucleoside triphosphates (dNTPs) *rTth* pol extends the annealed primer forming a complementary (cDNA) strand.

Following reverse transcription, the reaction mixture is re-heated, denaturing the RNA:cDNA hybrid. As the mixture cools, the upstream primer anneals to the cDNA strand and a double stranded DNA copy (or amplicon) of each target RNA sequence is synthesized catalysed by *rTth* pol. This first cycle of PCR is repeated over a number of cycles. Amplification occurs only in the region of the HIV-1 genome between the primers. The test amplifies and detects a 142 base target sequence located between position 905 and 1046 in a highly conserved region of the HIV-1 gag gene and defined by the primers IEP1B and IEP2A-bio respectively.

2.3.3 Detection

Amplified PCR products are detected in a microtitre-format enzyme-linked oligonucleotide assay (ELONA) (Whitby and Garson 1995). Following PCR amplification, the amplicons are bound to streptavidin coated micro-titre wells via a biotin extension on the 5'-end of the antisense primer. Bound products are then chemically denatured to form single stranded DNA by the addition of sodium hydroxide. The bound, single-stranded products are then hybridised to alkaline phosphatase labelled oligonucleotide probes specific for the target amplicons. Unhybridised probe is removed by washing and hybridised probe detected by a dioxetane-based chemiluminescent substrate (Lumiphos⁵³⁰; Lumigen Inc). Reactions

are read on a microtitre luminometer (Topcount, Canberra Packard Inc.) after one hour when the signal strength is maximal. Quantification and control of non-specific inhibition effects are achieved by the inclusion of three internal RNA controls at three concentrations. The controls contain the PCR primer binding sequences and regions for hybridisation with labelled probes distinct from the probe-binding region of the test sample. This allows the construction of an internal standard curve for each test sample from which the RNA load in the test sample can be read.

2.4 Protocol used

This protocol described, is for 12 samples (ten test samples, with a positive and negative control). Where different numbers of samples were used amounts of reagents were adjusted accordingly. To avoid RNAase degradation of the sample, RNA preparations were added to the PCR immediately after extraction, rather than storing RNA for amplification at a later date. For a list of reagents see Appendix 2.

2.4.1 Preparation of calibrators

The calibrators were taken from an Organon HIV-1 NASBA kit. The freeze dried calibrator was reconstituted with 0.55ml nuclease-free water, diluted to 5.5ml with RNA extraction lysis buffer, aliquotted in 40µl volumes and stored at -70°C.

2.4.2 Preparation of silica gel suspension

60g silica gel was suspended to 500ml in deionised water in a 500ml measuring cylinder and left at room temperature to settle for 24 hours. 430ml of the liquid was aspirated and more deionised water was added to 500ml, the silica re-suspended and left to settle for a further 5 hours. 440ml liquid was aspirated, the silica

re-suspended in the remaining liquid and to this added 600 μ l concentrated Hydrochloric acid. The suspension was sterilised by autoclaving (using liquids cycle) and aliquotted in 1ml volumes, ensuring that the silica was kept in suspension throughout, and stored at 4 $^{\circ}$ C.

2.4.3 RNA extraction and amplification

Lysis buffer and wash buffer were pre-warmed at 37 $^{\circ}$ C in a water-bath, to re-dissolve precipitated GuSCN, and each mixed thoroughly using roller apparatus to ensure GuSCN was completely dissolved. 33.3 μ l of calibrator were added to 12 ml lysis buffer (a ratio of 1:360) and 0.9ml of this mixture was aliquoted into 1.8ml Sarstedt vials (V-bottom) taking care to avoid contamination of the vial threads.

Plasma samples (semen or blood) were clarified by centrifuging at maximum speed for two minutes in a sealed rotor microfuge and 100 μ l of each sample of cleared plasma was added to separate vials of the lysis buffer/calibrator mix, vortexed and spun down briefly in a microfuge.

A suspension of silica gel was mixed thoroughly to ensure it was completely re-suspended and 50 μ l of this suspension was added to each vial. Each vial was vortexed thoroughly and incubated at room temperature for ten minutes in a vial shaker ensuring constant mixing of the silica suspension.

The vials were then spun by microfuging for approximately ten seconds at low speed (to spin down the silica gel) and the supernatant aspirated and discarded. 1ml of wash buffer was added to each vial and vortexed thoroughly to re-suspend the silica gel. This washing process was repeated, until the silica gel had been washed twice with wash buffer, twice with 70% ethanol and once with acetone. Care was taken to remove the last traces of acetone from each vial. The vials were incubated in a 56 $^{\circ}$ C

hot block for ten minutes with the caps removed to dry off the remaining acetone ensuring that all the vials were completely dry before proceeding.

50µl of elution buffer were added to each vial and the silica gel re-suspended by vortexing. The vials were tapped to shake the contents to the bottom and incubated again in a hot block at 56⁰C for ten minutes.

The reverse transcription/PCR mix (see below) was prepared during the elution period and 25µl aliquoted to each PCR reaction tube. The silica gel was spun down by centrifugation at maximum speed for two minutes on a microfuge and 25µl of the extracted RNA (in the supernatant) added directly to the reverse transcription/PCR mix, and mixed gently by pipetting. Each PCR reaction was then covered with two drops of oil and transferred to the PCR machine for amplification. The remaining RNA was stored immediately at -20⁰C, without removing the silica gel.

2.4.4 PCR mix (12 reactions)

120µl 5x reaction buffer (Perkin-Elmer EZ buffer)

96µl nucleotide mix (1.25mM each dNTP)

6µl primer IEP1B (66µg/ml stock)

6µl primer IEP2A-bio (66µg/ml stock)

24µl *rTth* polymerase

60µl manganese acetate (Perkin-Elmer)

25 µl sample (or sample + water)

2.4.5 PCR conditions

The following program was set up on the PCR machine and run.

1 cycle 60°C, 30 mins
4 cycles 94°C, 1min, 50°C, 1min, 72°C, 1min
35 cycles 94°C, 1 min, 57°C, 1 min, 72°C, 1min
1 cycle 72°C, 7 min
4°C indefinitely

Wells, coated with streptavidin (Sigma, Poole, UK) were washed twice with TTA wash buffer. 25µl of PCR product was added to 475µl of buffer A, mixed for 5 seconds and 100µl of this mixture added to each of four wells as shown in the plate map below (Figure 2.1). The plate was sealed with adhesive sealer and incubated at 45°C for 45 minutes in a humid box. Towards the end of this incubation period four probe solutions were made up (see below). After washing the wells 3 times with TTA, 100µl of 0.15M NaOH was added to each well and the plate incubated at room temperature for 5 minutes. The wells were washed 7 times with TTA.

2.4.6 Preparation and calibration of probe solutions

For each sample assayed four signals were measured, one for the unknown amount of test sample and three for the internal calibrators. The internal calibrators were made to give signals equivalent to 10^5 (Qa), 10^4 (Qb) and 10^3 (Qc) HIV-1 RNA copies/ml of sample.

The alkaline phosphatase-labelled probes, (Qa, Qb, Qc, IEL1) (Oswel, Southampton UK) were stored at -20°C in 50% glycerol to prevent degradation by freeze-thawing, were adjusted to a standard oligonucleotide concentration and used at a dilution of 1:1000 in assay buffer B.

100µl of each probe solution was added as shown in the plate map below (Figure 2.1), the wells sealed with an adhesive sealer and incubated at 45°C for 30 minutes in a humid box.

The wells were washed 16 times (2 x 8 washes) with TTA including a 1-minute soak after the eighth wash. 100µl Lumiphos substrate was added to each well. The wells were sealed with an adhesive sealer, and the plate incubated at room temperature for 60 minutes in the dark. The plate was read on a microtitre luminometer.

Figure 2.1 Plate map

QA	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	+ control
QB	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	+ control
QC	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	+ control
IEL1	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	+ control
QA	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	- control
QB	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	- control
QC	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	- control
IEL1	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	- control

2.4.7 Calculation and interpretation of results

For each sample assayed there are four signals, one for the unknown amount of test sample and three for the internal calibrators. The internal calibrators give signals equivalent to 10^5 (Qa), 10^4 (Qb) and 10^3 (Qc) HIV-1 RNA copies/ml of sample.

To calculate the value of the test sample a linear regression line was drawn between the three calibrators and the unknown value read from this line. These calculations were performed on an Excel spreadsheet programme which displayed the value of the unknown and the slope, intercept and goodness of fit (r^2 value) of the

regression line for each sample and control. Results were considered valid if the slope of the regression line was between 0.9 and 1.1 and the r^2 value was >0.90 . The positive control sample had been previously prepared and assigned a load of 12000 copies/ml based on its performance in commercial virus load assays (Roche Amplicor, Chiron Quantiplex). Assay runs were considered valid if the positive control gave a result within 0.3 log RNA copies/ml of its assigned value (6000 – 24,000 copies/ml). Samples giving results of $>10^6$ copies/ml were re-assayed at a dilution of 1:100 in negative human plasma as such results were outside the range of the assay. The cut-off of the assay was set at twice the value of the negative control.

2.5 Detecting Drug resistance mutations in blood and semen

To detect the presence of drug resistance associated mutations, HIV-1 protease (PR) and reverse transcriptase (RT; codons 1-235) genes from samples with detectable HIV-1 RNA were amplified by in-house nested RT-PCR and sequenced using the Beckman CEQ 2000 protocols (Beckman UK). Interpretation of reverse transcriptase and protease sequences was undertaken by uploading whole sequence information into the *Stanford HIV drug-resistance database*; <http://hivdb.stanford.edu/> (Shafer et al 2000).

2.6 Detecting HIV-1 proviral load in semen and first void urine

To assay concentrations of proviral DNA in the cellular component of semen and first void urine, stored cell samples were re-suspended in 1 ml of phosphate buffered saline and 100ul used for DNA extraction using a modified protocol with the QIAamp DNA mini Kit (Qiagen Ltd) (Qiagen Technical note.TS-QA04 01/99 2002). The DNA was eluted into 50µl of nuclease-free water and 1µl or 3µl of the elute used

for pro-viral DNA amplification and assay as described previously (Bennett et al 1999). Results were calculated as proviral DNA copies/ejaculate (or DNA copies/ml of urine). The limit of detection was 167 copies/ejaculate or 500 copies/ejaculate for 3 or 1µl input respectively. The assay was performed once only for each sample measured.

2.7 Comparison of NASBA and in-house RT-PCR for quantification of Semen plasma HIV-1 viral load

As part of a study conducted at Birmingham University into the penetration of antiretroviral drugs into the genital tract, a cohort of gay men infected with HIV-1 were invited to donate semen samples prior to starting combination antiretroviral therapy (Taylor et al 1999). HIV-1 RNA was extracted from seminal plasma samples using the method of Boom (Boom et al 1990) and quantified by NASBA (Dyer et al 1996). Residual seminal plasma was stored at -70°C. From these samples, ten were selected, representing a wide range of viral loads and transferred to the Department of Virology, University College London and analysed with the in-house RT-QPCR assay as described previously.

2.7.1 Results

The in-house method compared well with the NASBA method for assaying seminal plasma viral loads (see Table 2.1). One sample was inhibitory (sample number 9). On the whole, the in-house method gave values slightly higher than NASBA (median 3,930 Vs 1815 respectively; $p=0.02$, Wilcoxon rank test). There was excellent correlation between the two methods ($r=0.9440$; $p=0.0001$; Spearman's rank correlation, see Figure 2.2).

2.7.2 Discussion

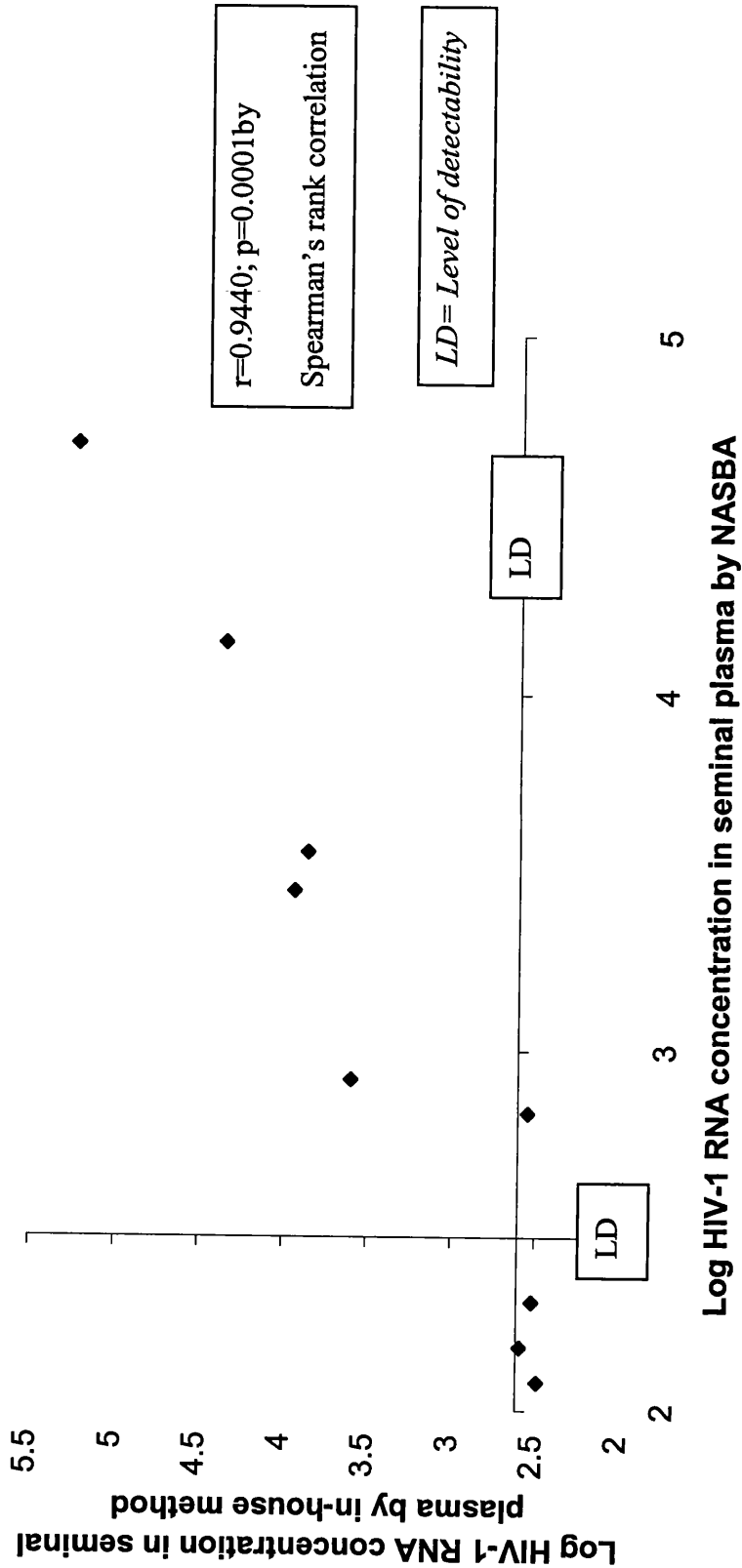
We have shown in this small comparative study of samples assayed by NASBA and the in-house method at UCL that there was good correlation between the two assays and that the in-house method gave values 0.5 log higher on average than NASBA.

Table 2.1 Comparison of NASBA and in-house RT-PCR for quantification of semen plasma HIV-1 RNA viral load

Sample No	RNA copies per ml by NASBA	RNA copies per ml by in-house method
1	14,000	22,628
2	670	<400
3	<200	<400
4	3,600	7,223
5	830	3,930
6	<300	<400
7	2,800	8,648
8	<300	<400
9	460,000	Inhibitory
10	50,000	174,741

The values achieved by the in-house assay were on the whole marginally higher than those found with NASBA (p<0.02).

Figure 2.2 Comparison of NASBA and in-house RT-PCR for quantification of semen plasma HIV-1 RNA viral load
 (values are in Log₁₀)



CHAPTER 3. THE EFFECTS OF URETHRITIS ON HIV-1 RNA LOAD AND PROVIRAL HIV-1 DNA IN THE SEMEN OF HIV POSITIVE MEN RECEIVING COMBINATION ANTIRETROVIRAL THERAPY

3.1 Aims

To investigate whether urethritis leads to an increase in SPVL and the detection of HIV-1 proviral DNA in semen cells in patients being treated with combination antiretroviral therapy.

3.2 Study Design

Homosexual men attending as outpatients, who had been receiving combination antiretroviral therapy for at least three months were eligible for the study. Exclusion criteria were any previous episode of urethritis or intercurrent illness in the month prior to study. Patients with symptomatic urethritis (cases) and those attending for a sexually transmitted infection screen in whom no diagnosis was found (controls) were recruited prospectively from the Mortimer Market Centre between November 1998 and March 2000. The patients with symptomatic urethritis attending the Department of Sexual Medicine (Birmingham Heartlands Hospital) were recruited episodically by one investigator between February 1999 and February 2000.

Written informed consent was obtained from all patients and clinical and demographic data collected. A urethral smear and culture for diagnosing gonorrhoea, a urethral swab for diagnosing chlamydia using ligase chain reaction (Abbott Diagnostics, Abbot Park, IL, USA) and blood for viral load were taken on their first visit. In those negative for gonorrhoea and chlamydia, non-specific urethritis was defined as those patients symptomatic for urethritis with five or more polymorphs per

high power field (p/hpf) on microscopy or asymptomatic patients with 10 or more p/hpf on microscopy. Patients then provided a semen sample by masturbation into a sterile container. Patients were asked to collect the entire volume of the ejaculate into sterile containers. Most patients provided semen samples prior to voiding urine.

Those patients diagnosed with urethritis, whether gonococcal or non-gonococcal, were treated with appropriate antibiotics. Cases and controls attended two weeks later for repeat smears, gonococcal culture, blood and semen samples. Most patients also provided samples one week after the first visit. Blood and semen samples were assayed for HIV-1 RNA load in plasma by the methods described in chapter 2. At all visits sexual histories were taken to exclude further exposure to sexually transmitted infections.

3.3 Statistical analysis

Eligible patients whom were offered participation in the study at the Mortimer Market Centre were compared with those whom were not, and among those offered, those who agreed to participate were compared with those who declined. These comparisons were performed separately among cases and controls with respect to age and stage of disease. In the study population cases were compared with controls with respect to the most recent CD4⁺ T-cell count prior to first visit, and CD4⁺ T-cell count and BPVL prior to ever starting combination antiretroviral therapy. For comparisons of age the t-test was used and for CD4⁺ T-cell counts and viral loads the Mann-Whitney test was used. The χ^2 test for trend was used for HIV disease stage. The χ^2 test was also used to test for an association between the detection of HIV RNA in semen and blood. Fisher's exact test was used to test for an association between detectability of proviral DNA in semen and presence of urethritis.

3.4 Results

Of 84 eligible patients at the Mortimer Market Centre, 47 (60%) were offered entry into the study and of these, 36 (77%) agreed to take part. A comparison within control and case groups, between those offered and not offered entry into the study, showed there was no significant difference in age ($p=0.154$ and $p=0.550$ respectively) or stage of HIV infection ($p=0.345$ and $p=0.799$ respectively). In those offered entry into the study there was no difference, between those recruited or not, in the age of controls and cases ($p=0.117$ and $p=0.295$ respectively) nor in the stage of HIV infection among controls ($p=0.829$). Patients with symptomatic urethritis however, appeared to be more likely to decline study participation if they had more advanced HIV disease stage ($p=0.043$). (Data not shown).

Samples from 16 control patients and 24 cases (ten with gonorrhoea, six with chlamydia and eight with non-specific urethritis) were collected. Baseline characteristics of all patients, and differences between cases and controls are shown in Table 3.1.

The majority of patients had undetectable BPVLs a few months prior to the study. Both the median nadir $CD4^+$ T-cell count before antiretroviral therapy was started and current $CD4^+$ T-cell count just prior to the study were similar in cases and controls. All participants were on at least three antiretroviral drugs (see Tables 3.2 and 3.3).

3.4.1 Seminal plasma HIV-1 RNA loads

In 13 of 16 controls and 18 of 24 cases on antiretroviral therapy HIV RNA was undetectable in semen at visit 1 (Figures 3.1 and 3.2). At visit 1 there was a strong association between detectability of HIV-1 RNA in seminal plasma with that

in blood plasma in cases ($p < 0.0005$) and an association that was not significant in controls ($p = 0.06$) (Table 3.4). In those with detectable BPVL at visit 1 0/5 cases compared with 4/7 controls had undetectable SPVLs at visit 1 ($p = 0.08$, Fisher's exact test). Median BPVLs were non-significantly higher in the cases compared to controls at visit 1 (69,000 cps/ml vs 1,052cps/ml; $p = 0.062$, Mann-Whitney two-sided test).

Among controls, consistent with earlier studies in patients on combination antiretroviral therapy, when virus was undetectable in blood it was undetectable in seminal plasma and low BPVLs (ranging from 600-11,000 copies/ml) were associated with low or undetectable SPVLs. Also in line with earlier studies, some controls with undetectable BPVLs and SPVLs had detectable proviral DNA in semen despite most of them being on suppressive therapy for more than 1 year (Table 3.2). Time on antiretroviral therapy ranged from 2 to 42 months for controls.

Among cases with undetectable BPVL prior to recruitment BPVL and SPVL remained undetectable at visit 1 and subsequent visits in 5/5 episodes of chlamydial urethritis (CU), 6/7 episodes of non-specific urethritis (NSU) and 4/5 cases of gonococcal urethritis (GU). In two cases (patients 23 and 24 with GU and NSU respectively) with undetectable BPVL just prior to the first study visit there were low to moderate SPVLs (5928 and 1512 copies/ml respectively) which became undetectable by visit 2 following treatment for urethritis. The reduction in SPVL in the second of these cases however, may be accounted for by assay variability (Table 3.3).

Of 7 cases with detectable BPVL prior to recruitment 3 (1 GU, 1 CU and 1 NSU) had undetectable BPVLs and SPVLs at visit 1. These results were maintained at subsequent visits apart from patient 4 who had low levels of detectable RNA in blood at visits 2 and 3. (In patient 5, because of difficulties with diluting the sample,

the limit of detection in semen at visit 1 was 10,000 copies/ml). Four cases of urethritis (patients 19-22, all with GU) had poor control of BPVL prior to presentation with their urethritis (and at visit 1) and all of them had high SPVLs. In one of these cases in particular, the SPVL was higher than the BPVL but was reduced 20-fold following treatment for gonorrhoea (patient 22, Table 3.3). Time on antiretroviral therapy for cases ranged from 7 to 51 months for cases.

In patients 19-21, (with gonococcal urethritis) HIV-1 RNA was detectable in seminal plasma at lower concentration than in blood and these loads did not change significantly following treatment for gonorrhoea. Two of these patients (20 and 21) voided urine prior to providing semen samples and the first catch of urine from patient 20 was retained at visit 1 and follow-up. It was not possible to amplify the amount of HIV-1 RNA in cell free urine from this patient because of inhibitors. However HIV-1 proviral DNA was found in urinary cells at visit 1 but was not detected at follow up (data not shown). Patient 21 was the only one in this group who had detectable HIV-1 proviral DNA in semen at the first visit and this remained detectable following treatment.

3.4.2 Detection of semen HIV-1 proviral DNA

Seminal cell pellets were examined for proviral DNA in patients recruited from Mortimer Market only (Tables 3.2 and 3.3). At visit 1, 15/19 cases and 15/16 controls had undetectable DNA provirus in their ejaculate ($p=0.35$; see Tables 2, 3). Among those with undetectable seminal plasma viral load at visit 1, 12/14 cases and 12/13 controls had undetectable proviral DNA in semen at visit 1 ($p=1.00$)

In 12 cases with undetectable SPVLs at visit 1, semen samples were available for measuring proviral HIV-1 DNA both at the first visit and following treatment for

urethritis. In 10 of these cases provirus was undetectable during urethritis and following treatment remained undetectable in eight. In two cases provirus was detectable during urethritis and following treatment remained detectable in one case but was undetectable in the other.

In cases 23 and 24, in whom SPVLs were reduced following treatment for urethritis HIV-1 provirus was not detected in semen at visit 1, a finding not inconsistent with their respective low BPVLs and SPVLs (Tachet et al 1999). Unfortunately samples were not available for measuring HIV-1 proviral DNA in patient 22.

3.5 Conclusions

This study provides important evidence that antiretroviral therapy may not only reduce sexual transmission of HIV-1 by reducing viral load in semen but may also reduce the potential facilitating effects of sexually transmitted infections on HIV-1 transmission. However, the observations also suggest that at least in some patients antiretroviral therapy may not always control the potentiating effects of urethritis on HIV replication in the genital tract. This study also confirms that in patients whose BPVLs may be undetectable for more than 1 year and whose SPVLs are also undetectable, HIV-1 provirus may still be detectable in semen (Liuzzi et al 1999; Tachet et al 1999; Zhang et al 1998) as well as in blood (Demeter et al 2002).

Table 3.1 Baseline Characteristics of patients recruited

	N	Mean Age Years (S.D.)	Median Baseline BPVL pre CART (range)	Median BPVL pre study+ (range)	Median Nadir CD4 pre CART (range)	Median CD4 pre study+	Median Time on CART Months (range)
Urethritis	24	36.8 (6.2)	101,000 (8047 – 1,480,769)	<50 (<50 – 156,000)	195 (20-340)	410 (80-900)	17.5 (3 – 51)
No Urethritis	16	39.1 (4.9)	104,300 (10500 – 262049)	<50 (<50 – 47700)	150 (10–340)	310 (100-900)	16 (2 – 42)
<i>P</i>		<i>0.24</i>	<i>0.64</i>	<i>0.88</i>	<i>0.62</i>	<i>0.61</i>	<i>0.63</i>

CART = combination antiretroviral therapy

BPVL= blood plasma viral load : copies/ml

CD4 : x10⁶/l.

+ = most recent value before study entry (within 3 months)

Table 3.2 Plasma viral load (BPVL), semen viral load (SPVL) and detection of semen proviral HIV-1 DNA in controls

Patient Number	Current ART	BPVL RNA copies/ml			SPVL RNA copies/ml			Semen Proviral DNA		
		Pre-Visit 1	Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3	Visit 1	Visit 3
		Visit 1								
101	DDC/3TC/IND	<50	<500	<500	<500	<1000	<1000	<1000	+	-
102	AZT/3TC/NVP	<50	<500	<500	<500	<1000	<1000	<1000	-	+
103	DDI/3TC/EF/RIT/IND	<50	<500	N	<500	<1000	N	<1000	-	+
104	AZT/3TC/DDI	<50	<500	<500	<500	<1000	<1000	<1000	-	+
105	D4T/DDI/EF	60	<500	<500	<500	<1000	<1000	<1000	-	-
106	D4T/RIT/SAQ	<50	<500	<500	<500	<1000	<1000	<1000	-	-
u107	AZT/3TC/EF	<50	<500	<500	<500	<1000	<1000	<1000	-	+
u108	NLF/3TC/NVP	<50	<500	<500	<500	<1000	<1000	<1000	-	+
109	D4T/DDI/EF	<50	<500	<500	<500	<1000	1,288	<1000	-	-
110	AZT/3TC/SAQ	200	664	N	702	2,716	N	1,300	-	N
111	AZT/3TC/IND	<50	744	N	N	2,116	N	N	-	N
u112	NLF/3TC/ABC	<50	832	N	<500	<1000	N	<1000	-	-
113	D4T/3TC/NLF	<50	1,052	N	N	1,068	N	N	-	N
u114	D4T/DDC/NLF	100	2,624	N	1,894	<1000	N	<1000	-	+
u115	D4T/3TC/EF	5,700	9,039	3,946	3,913	<1000	<1000	<1000	-	+
u116	DDI/NLF/SAQ	100	11,654	10,876	6,910	<1000	<1000	<1000	-	-

u = patients voiding urine before producing semen samples. N = not done + = detected, - = not detected

IND = Indinavir; NVP = Nevirapine; EF= Efavirenz; RIT = Ritonavir; SAQ = Saquinavir; NLF = Nelfinavir; ABC = Abacavir

Table 3.3 Plasma viral load (BPVL), semen viral load (SPVL) and detection of semen proviral HIV-1 DNA in cases

Patient Number	Current ART	STI	BPVL (RNA copies/ml)						SPVL (RNA copies/ml)						Semen Proviral DNA		
			Pre	Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 1	Visit 2	Visit 3
			Visit 1	Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 1	Visit 2	Visit 3
1	AZT/3TC/EF	NSU	<50	<500	<500	<500	<500	<500	<500	<1000	<1000	<1000	<1000	<1000	-	-	-
2	3TC/RIT/SAQ	C	<50	<500	<500	<500	<500	<500	<500	<1000	<1000	<1000	<1000	<1000	-	-	-
3	D4T/3TC/RIT	NSU	<50	<500	<500	<500	<500	<500	<500	<1000	<1000	<1000	<1000	N	-	-	N
4	AZT/3TC/NVP	G	5,200	<500	1268	1429	<500	<500	1429	<1000	<1000	<1000	<1000	<10000	-	-	-
5	AZT/DDI/EF	NSU	1,500	<500	<500	N	<500	<500	N	<10000	<1000	<1000	N	N	-	+	+
"6	D4T/3TC/IND	C	<50	<500	N	<500	<500	N	<500	<1000	N	<1000	<1000	<1000	-	-	-
"7	D4T/DDI/NLF	NSU	<50	<500	<500	<500	<500	<500	<500	<1000	<1000	<1000	<1000	<1000	-	-	-
"8	AZT/3TC/IND	NSU	<50	<500	<500	N	<500	<500	N	<1000	<1000	<1000	N	N	+	+	+
"9	AZT/3TC/RIT/IND	C	1,000	<500	<500	<500	<500	<500	<500	<1000	<1000	<1000	<1000	<1000	-	-	-
"10	AZT/3TC/NLF	NSU	<50	<500	<500	<500	<500	<500	<500	<1000	<1000	<1000	<1000	<1000	-	-	-
"11	D4T/3TC/SAQ/NLF	C	<50	<500	<500	<500	<500	<500	<500	<1000	<1000	<1000	<1000	<1000	N	-	+
"12	D4T/3TC/RIT/SAQ	NSU	<50	<500	<500	<500	<500	<500	<500	<1000	<1000	<1000	<1000	<1000	-	-	+
13	AZT/3TC/RIT/SAQ	G	<50	<500	<500	<500	<500	<500	<500	<1000	<1000	<1000	<1000	<1000	+	+	-
14	AZT/3TC/EF	G/C	<50	<500	N	N	<500	N	N	<1000	N	N	N	N	-	-	N
15	AZT/3TC/NLF	G	<50	<500	<500	<500	<500	<500	<500	<1000	<1000	<1000	<1000	<1000	-	-	-
16	D4T/3TC/NVP	G	<50	<400	N	<400	<400	N	<400	<400	N	<400	<400	<400	N	N	N
17	D4T/3TC/EF	C	<50	<400	N	<400	<400	N	<400	<400	N	<400	<400	<400	N	N	N
18	D4T/3TC/NVP/HU	C	<50	<400	N	<400	<400	N	<400	<400	N	<400	<400	<400	N	N	N
19	DDI,ABC,NLF,SAQ	G	17,900	13,913	16,257	11,249	13,913	16,257	11,249	6,412	3,962	4,012	6,412	4,012	-	-	-
"20	AZT,3TC,DDC	G	156,000	98,882	35,324	85,262	98,882	35,324	85,262	15,770	23,558	23,786	15,770	23,786	-	-	+
"21	AZT,3TC,DDI	G	58,200	85,470	44,158	49,647	85,470	44,158	49,647	26,422	14,523	21,916	26,422	21,916	+	+	+
22	D4T,ABC,NVP	G	5,300	69,000	N	49,000	69,000	N	49,000	100,000	N	4,300	100,000	4,300	N	N	N
23	D4T,3TC,NLF,SAQ	G	<50	713	<500	986	713	<500	986	5,928	<1000	<1000	5,928	<1000	-	-	-
24	D4T,3TC,NVP	NSU	<50	<500	<500	<500	<500	<500	<500	1,512	<1000	<1000	1,512	<1000	-	-	-

Legend for Table 3.3

STI = sexually transmitted infection	G = gonorrhoea	C = chlamydia	NSU = non-specific urethritis
u= passed urine before providing semen sample	* = visit 2	N = not done	+ = detected - = not detected
IND = Indinavir; NVP = Nevirapine; EF= Efavirenz; RIT = Ritonavir; SAQ = Saquinavir; NLF = Nelfinavir; ABC = Abacavir;			
HU = Hydroxyurea			

Table 3.4 Association between semen viral load (SPVL) and blood viral load (BPVL) at visit 1 in cases and controls

		Cases		Controls	
		BPVL		BPVL	
SPVL	+ve	+ve	-ve	+ve	-ve
	-ve	5	1	3	0
		0	18	4	9

P < 0.0005 for cases. P = 0.06 for controls

+ve = detectable -ve = undetectable

For most cases the limit of detection for RNA was 500 copies/ml for blood and 1000 copies /ml for semen

For 4 cases the limit of detection of RNA was 400 copies/ml for blood and 400 copies /ml for semen

The values are not affected by using these different limits.

Figure 3.1 Plot of \log_{10} seminal plasma HIV-1 RNA versus \log_{10} blood plasma HIV-1 RNA in patients without urethritis (n=16)

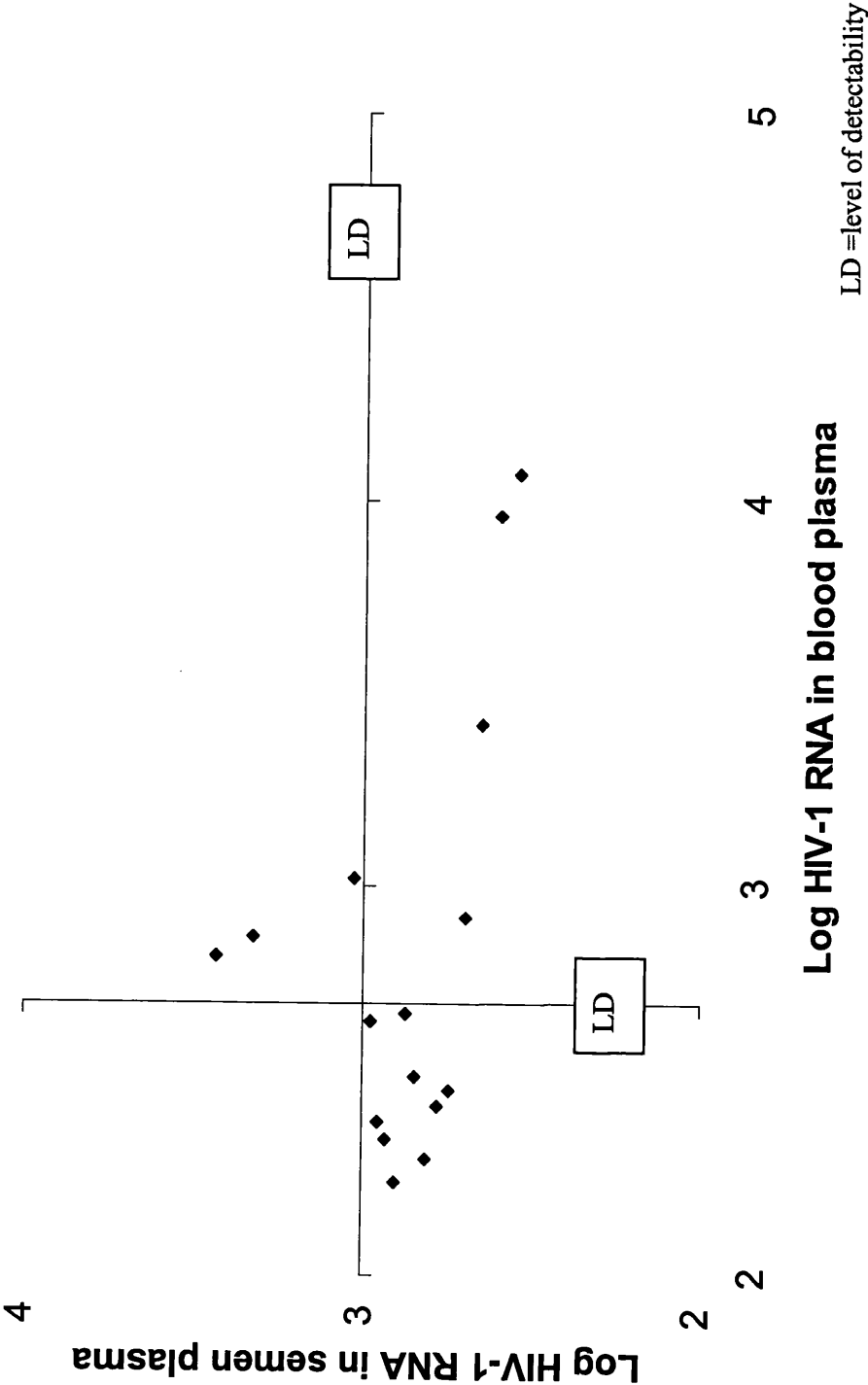
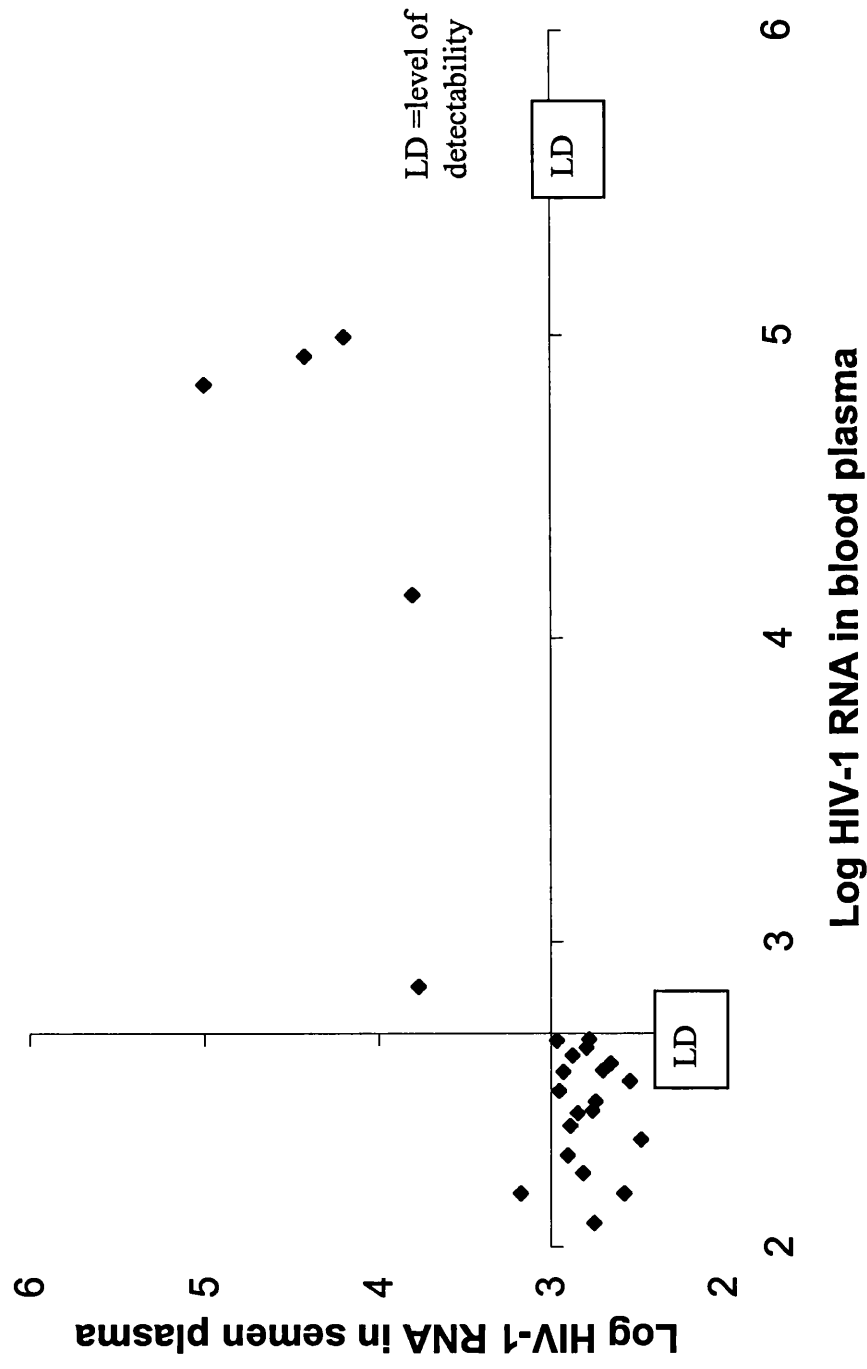


Figure 3.2 Plot of \log_{10} seminal plasma HIV-1 RNA versus \log_{10} blood plasma HIV-1 RNA in patients with urethritis (n=24)



CHAPTER 4. DRUG-RESISTANT HIV-1 IN THE SEMEN AND BLOOD OF MEN RECEIVING ANTIRETROVIRAL THERAPY WITH URETHRITIS

4.1 Introduction

We demonstrated in the prospective case controlled study in chapter 3, that in patients on maximally suppressive combination ART, urethritis had minimal effect on SPVL whereas in some patients on poorly suppressive combination ART, SPVL appeared to be higher during STIs, (Sadiq et al 2002). Here we report results of drug resistance analyses of semen and blood virus in the cases with STIs on failing antiretroviral regimes.

4.2 Methods

Full details of the study design and methodology employed are described in chapter 3. This chapter describes the virological characteristics of the cases within the case-control study. Unfortunately, insufficient nucleic acid extract or original sample were left for re-assessing viral load by ultrasensitive assays, which became available during the study. Further cell fractions were not available for proviral DNA analysis. To detect the presence of drug resistance associated mutations, HIV-1 protease (PR) and reverse transcriptase (RT; codons 1-235) genes from samples with detectable HIV-1 RNA were amplified by in-house nested RT-PCR and sequenced using the Beckman CEQ 2000 protocols (Beckman UK). Interpretation of RT and protease PR sequences was undertaken using the database maintained at Stanford University (Shafer et al 2000).

4.3 Results

As reported in chapter 3, in 19 patients with undetectable BPVL during acute urethritis, all maintained undetectable SPVL apart from one in whom SPVL was low and close to the level of detectability. We were unable to PCR-amplify the RT and PR genes from semen virus in this patient. In all five of the 24 cases with detectable BPVL during urethritis, SPVL was detectable. In two of these patients SPVL was higher than BPVL and following antibiotic treatment SPVL was reduced to below BPVL (Table 4.1). In four of these five cases, we were able to PCR-amplify the RT and PR genes from both blood and semen virus. In three of these four, in whom SPVL was high but did not change following treatment for urethritis, key (primary) drug resistance-associated mutations were identified in semen plasma virus, which matched those in the corresponding blood plasma virus. In one of these three (case 19) multiple mutations in RT and PR were detected, whereas RT mutations alone were identified in the other two cases. These changes conferred high level resistance to a range of currently licensed antiretroviral drugs (Table 4.1). No differences were observed in these mutational patterns between the first and second visits. In patient 22 SPVL was significantly higher during gonorrhoea than following its treatment (5 vs 3.6 log copies/ml respectively) while BPVL remained unchanged (Figure 4.1). Key mutations conferring high level resistance to non-nucleoside and nucleoside analogue reverse transcriptase inhibitors were identified in the blood and semen of this patient at visit 1, but amplification of semen virus at the second visit was not possible. The small semen sample volume and low viral loads identified in three of 16 controls with detectable semen viral load precluded generation of nucleotide sequence from these viruses.

4.4 Conclusion

These data suggest that in some individuals without maximally suppressed blood plasma viral load, the combination of STI's and ART may actually promote the transmission of drug resistant virus.

Table 4.1 Drug resistant HIV-1 in seminal plasma of men with concurrent acute sexually transmitted infections (STI)

Case	Antiretroviral Therapy	STI ¹	BPVL (log ₁₀ copies/ml)		SPVL (log ₁₀ copies/ml)		Resistance Associated Mutations in blood and semen virus at both visits ²	REDUCED DRUG SUSCEPTIBILITIES ³
			Visit 1	Visit 2	Visit 1	Visit 2		
19	ddI, ABC, NfV, SQV	GU	4.1	4.1	3.8	3.6	RT: M41L, L74V, V118I, M184V, T215Y PR: L10I, K20I, L24I, M36I, M46L, I54V, L63P, A71V, T74S, V82A	ZDV, d4T: low level resistance ddI, ddC, ABC, 3TC: high-level resistance APV, SQV: intermediate resistance IDV, RTV, NfV: high-level resistance ZDV, d4T, ddI, ddC: intermediate resistance ABC, 3TC: high-level resistance EFV, NVP, DLV: potential low level resistance ZDV, ABC, 3TC: high-level resistance ddI, d4T, ddC: intermediate resistance
20	ZDV, 3TC, ddI	GU	5	4.9	4.2	4.4	RT: M41L, A98G, M184V, L210W/L, T215Y	ABC, 3TC: high-level resistance EFV, NVP, DLV: potential low level resistance ZDV, ABC, 3TC: high-level resistance ddI, d4T, ddC: intermediate resistance
21	ZDV, 3TC, ddI	GU	4.9	4.7	4.4	4.3	RT: M41L, E44A, D67N, K70R, M184V, T215Y, K219E	ABC: low level resistance ddI, 3TC, TDF: intermediate resistance ddC: high level resistance EFV, NVP, DLV: high-level resistance NA
22	D4T, ABC, NVP	GU	4.8	4.7	5	3.6	*RT: K65R, K103N, Y181C	ABC: low level resistance ddI, 3TC, TDF: intermediate resistance ddC: high level resistance EFV, NVP, DLV: high-level resistance NA
23	d4T,3TC, NfV,SQV	GU	2.9	3	3.8	<3	NA	ABC: low level resistance ddI, 3TC, TDF: intermediate resistance ddC: high level resistance EFV, NVP, DLV: high-level resistance NA
24	D4T, 3TC, NVP	NSU	<2.7	<2.7	3.2	<3	NA	ABC: low level resistance ddI, 3TC, TDF: intermediate resistance ddC: high level resistance EFV, NVP, DLV: high-level resistance NA

Legend for Table 4.1

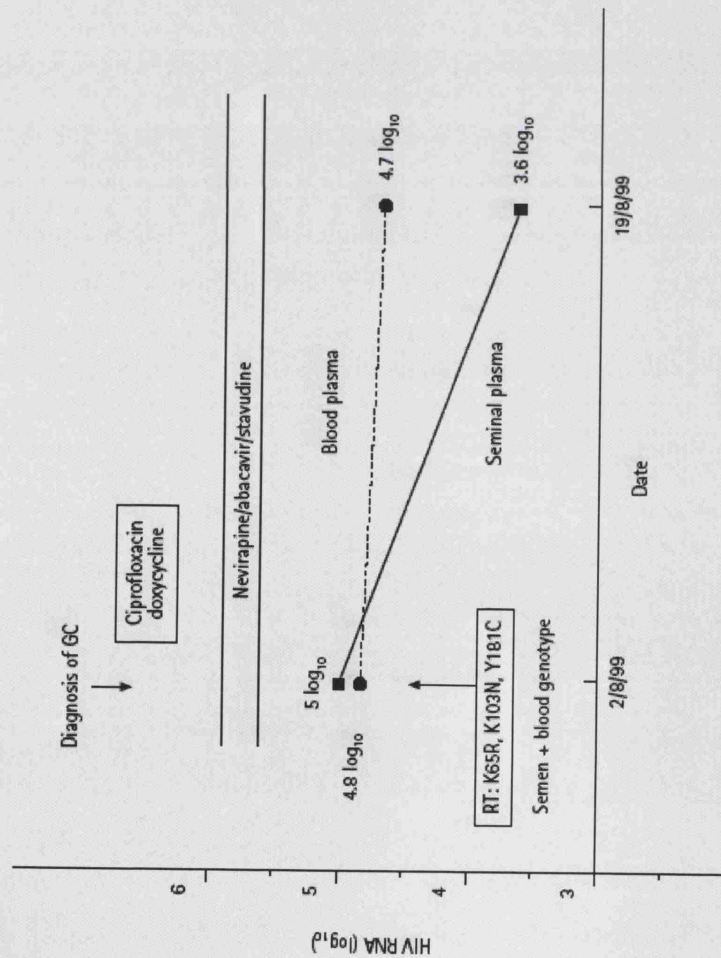
¹GU=Gonococcal urethritis. NSU=Non specific urethritis. BPVL= blood plasma viral load. SPVL= seminal plasma viral load. NA = Not amplifiable; sample was of insufficient volume for viral load analysis or viral load was too low to amplify RT and PR for sequencing

²RT=reverse transcriptase. PR=protease.

³d4T=stavudine. 3TC=lamivudine. ZDV= zidovudine. NVP=nevirapine. NFV=nelfinavir. SQV=saquinavir. ABC=abacavir. ddI=didanosine. ddC= zalcitabine. APV=amprenavir. DLV=delavirdine. TDF=tenofovir. IDV=indinavir. RTV=ritonavir. EFV=efavirenz. ddI=didanosine.

* sequence not obtained from semen virus at 2nd visit

Figure 4.1 Effect of treating gonococcal urethritis on blood and semen plasma HIV-1 RNA with failing ART and drug-resistant virus



Patient 22 presented with gonorrhoea and a concurrent seminal plasma viral load of 100,000 copies / ml. Genotypic analysis of extracellular virus from blood and semen demonstrated the presence of reverse transcriptase mutations K65R, K103N, Y181C, in both blood and semen, associated with reduced susceptibility to abacavir, tenofovir, and all non-nucleoside reverse transcriptase inhibitors. Treatment with ciprofloxacin 500 mg and doxycycline 100 mg twice daily for 7 days brought about approximately 1.4 log₁₀ reduction in seminal plasma viral load after 17 days, whereas blood plasma viral load did not alter significantly.

CHAPTER 5. THE EFFECTS OF URETHRITIS ON SEMINAL PLASMA HIV-1 RNA LOADS IN HOMOSEXUAL MEN NOT RECEIVING ANTIRETROVIRAL THERAPY

5.1 Aims

To study the effects of urethritis on SPVL in homosexual men with HIV infection who were not receiving antiretroviral therapy.

5.2 Methods

HIV-1 infected homosexual men not receiving ART for at least three months attending two UK sexual health clinics either with urethritis (cases) or for a sexual health check-up but no STI (controls) were recruited prospectively between November 2000 and October 2002 (study visit 1). Patients were excluded if they had an episode of urethritis or systemic illness in the previous month. Participants had routine urethral swabs for gonorrhoea (by microscopy and culture), chlamydia (using ligase chain reaction; Abbott Diagnostics, Abbot Park, IL, USA) and non-gonococcal urethritis (NGU) by microscopy. For the study, in those who were negative for gonorrhoea on microscopy, NGU was defined as those patients symptomatic for urethritis with five or more polymorphs per high power field (p/hpf; a magnification of X 1000) on microscopy or those asymptomatic patients with 10 or more p/hpf on microscopy. This definition was used because of our observation that consistency of microscopy alone for predicting NGU is poor when polymorph counts are low (Smith R et al 2003). The initial diagnosis of NGU was changed to non-specific urethritis (NSU) if subsequent chlamydia and gonococcal culture tests remained negative. In addition to serological tests for syphilis taken on their first visit, blood was also

collected for HIV-1 RNA quantification and patients then provided a semen sample by masturbation into a sterile container. All patients provided semen samples prior to voiding urine and were advised against using lubricants during masturbation.

Patients diagnosed with urethritis, whether GU or NGU, were treated with appropriate antibiotics. Cases and controls were asked to attend the following week (visit 2) and two weeks later (visit 3) for repeat smears, gonococcal culture and blood and semen samples. At all visits, clinical and demographic data were collected including sexual histories.

A sample size of 20 cases and controls was required to give approximately 80% power to detect as significant a difference in mean log-SPVL at first visit of 0.7 (i.e. a 5-fold difference in SPVL), as observed previously in Africa, relative to a standard deviation of measurements in each group of 0.8, and taking the standard 5% significance level. It was decided to try to recruit more controls to increase this power.

5.2.1 Virology Methods (see chapter 2)

Semen and blood samples were centrifuged within two hours of collection and the plasma and cellular components stored at -70°C. HIV-1 RNA was extracted from blood and semen plasma by a silica gel capture method previously observed to successfully remove inhibitors of PCR (Boom et al 1990) and quantified using an in-house, internally calibrated reverse transcribed PCR assay (RT-QPCR, Department of Virology UCL). The lower limit of quantification was 1000 copies/ml.

5.3 Statistical analysis

Cases were compared with controls with respect to age, years since HIV diagnosis, ethnicity, median numbers of partners in previous 3 months, and most

recent CD4⁺ T-cell count and HIV-1 viral load prior to first visit. For comparisons of age, number of partners and time since HIV diagnosis the Mann-Whitney test was used. For CD4⁺ T-cell counts and log viral loads prior to first visit, and also at first visit, the t-test was used. In all analysis undetectable HIV-1 RNA loads were considered as 500 copies/ml (half the limit of detection), and log₁₀ values were used for analysis of both HIV-1 RNA loads and CD4⁺ T-cell counts. To compare ethnicity and HIV-1 RNA detectability at visit 1 Fisher's exact test was used. To compare HIV-1 RNA loads in blood and semen within patients at visit 1 the paired t-test was used, and their correlation assessed using Pearson's correlation coefficient. Average changes in HIV-1 RNA loads across study visits were estimated for cases and controls, and these changes compared. This analysis was based on generalised estimating equations (GEE) of STATA 7, because of multiple measurements for patients. As planned subgroup analysis, comparisons with controls were made for all cases, for NSU cases alone, and cases with chlamydial urethritis (CU) or GU.

5.4 Results

20 cases (9 GU, 3 CU, 1 combined CU and GU and 7 NSU) and 35 controls were recruited. In this study, all cases had polymorph counts of >10 p/hpf counts and all controls counts of <5/hpf. All cases were symptomatic, except one with NSU who had a polymorph count of 11 p/hpf. Three of the remaining NSU cases had polymorph counts of between 10 and 20 p/hpf and the other three, counts of >20/hpf. All cases of CU or GU had polymorph counts of >20 p/hpf except one with GU with a count of 15 p/hpf. Seven controls had symptoms of urethral discomfort, but were negative for chlamydia and gonorrhoea. All of these patients had held their urine for at least three hours prior to having urethral swabs done. One case with GU and two controls were

receiving antibiotics for unrelated reasons at presentation. The case with GU had been on his fourth day of phenoxymethyl-Penicillin for tonsillitis, one control was being maintained on cotrimoxazole for PCP prophylaxis and the other control had been taking low dose erythromycin for folliculitis. This latter patient was not symptomatic for urethritis. Median age, years since HIV diagnosis, ethnicity, numbers of sexual partners in the previous 3 months, pre-study BPVL and pre-study CD4 count were similar between cases and controls (Table 5.1).

5.4.1 BPVLs, SPVLs at study visit 1 and follow-up (Table 5.1, Figures 5.1, 5.2 & 5.3)

Visit 1. Using a level of detection of 1000 copies/ml HIV-1 RNA was detectable in 16/20 cases compared with 23/35 controls in semen ($p=0.36$, Fisher's exact test) and in 18/20 cases compared with 33/35 controls in blood ($p=0.62$). BPVLs were higher than SPVLs in controls by 0.66 log ($p < 0.001$) and there was a fairly good correlation between BPVL and SPVL ($r=0.46$, $p=0.005$ Pearson coefficient). Among cases overall and in patients with GU or CU, BPVLs were similar to SPVLs ($p=0.58$ and $p=0.52$ respectively, paired t-test) and there was again a good correlation between BPVL and SPVL ($r=0.61$, $p=0.004$ and $r=0.71$, $p=0.006$ respectively). SPVLs were non-significantly lower than BPVLs, in those with NSU, by 0.52 log ($p=0.07$).

At visit 1, there was no difference in mean log BPVL between cases and controls. Compared with controls mean log SPVL appeared higher in cases overall, (3.99 log for cases v 3.55 log for controls; $p=0.08$), significantly higher in GU/CU cases (4.27 log; $p=0.014$) but were similar in NSU cases (3.48 log; $p=0.82$) (see Table 5.1 and Figure 5.3). No difference was detected either in SPVL or BPVL in cases of

CU compared with GU (mean BPVL: 4.5 log vs 4.07 log, $p=0.266$; mean SPVL: 4.58 log vs 4.15 log; $p=0.44$, respectively).

Follow-up. 24 controls and 21 cases attended at least one follow-up visit. No difference was detected in mean BPVL or SPVL between cases overall and controls at follow up. Among those with CU/GU, mean SPVL remained approximately half a log higher compared with controls but this difference was not significant.

5.4.2 Changes in log viral loads from visit 1 to follow-up (Figure 5.4)

No significant changes in BPVL or SPVL from visit 1 to follow-up were detected in cases overall, or controls. However, among those with GU/CU alone, SPVLs, but not BPVLs, decreased following antibiotic treatment by on average 0.25 log (95% CI 0.03 – 0.47, $p=0.028$). When compared with the changes observed among controls, this effect appeared to be broadly maintained with a relative reduction in SPVL in GU/CU cases of 0.34 log (-0.01 – 0.68; $p=0.056$). No change in SPVL was observed in those with NSU alone.

5.5 Conclusions

These results indicate GU and CU, though not NSU, increase SPVL in those not on ART and that in the small number of patients with chlamydial infection, the effect on SPVL appeared to be just as pronounced as those with gonorrhoea.

Table 5.1 Baseline characteristics and viral loads of cases and controls

	Urethritis	Controls	p**
N	20 (9 GU, 1 GU/CU, 3 CU, 7 NSU)	35	-
Median age in years (range)	33.3 (23.9 – 48.5)	35.6 (24.5 – 61.8)	0.345
Years since HIV diagnosis (range)	3.36 (0.41 – 14.18)	1.13 (0.11 – 16.19)	0.069
White ethnicity (n)	18	31	1.00
Median partners in last 3 months (range)	4 (1-21)	3 (0-51)	0.297
Mean pre-study log ₁₀ BPVL (95% CI)	4.26 (3.90 – 4.62)	4.34 (4.06 – 4.63)	0.728
Mean pre-study log ₁₀ CD4 count (95% CI)	2.63 (2.48 – 2.78)	2.63 (2.52 – 2.74)	0.970
BPVL at study visit 1			
Comparison of all urethritis with controls	4.11 (3.76 – 4.45)	4.21 (4.03 – 4.40)	0.550
GU/CU only	4.17 (3.75 – 4.59)	-	0.821
NSU only	4.00 (3.22 – 4.77)	-	0.385
BPVL after study visit 1			
Comparison of all urethritis with controls	4.19 (3.78 – 4.59) (n=22/15*)	4.27 (4.02 – 4.52) (n=23/21*)	0.752
GU/CU only	4.38 (3.90 – 4.87) (n=13/9*)	-	0.657
NSU only	3.92 (3.24 – 4.60) (n=9/6*)	-	0.306
SPVL at study visit 1			
Comparison of all urethritis with controls	3.99 (3.53 – 4.45)	3.55 (3.27 – 3.83)	0.078
GU/CU only	4.27 (3.66 – 4.87)	-	0.014
NSU only	3.48 (2.78 – 4.17)	-	0.820
SPVL after study visit 1			
Comparison of all urethritis with controls	3.88 (3.54 – 4.23) (n=21/16*)	3.59 (3.24 – 3.94) (n=24/23*)	0.228
GU/CU only	4.12 (3.54 – 4.69) (n=12/10*)	-	0.111
NSU only	3.52 (3.39 – 3.66) (n=9/6*)	-	0.823

GU = gonococcal urethritis; CU = Chlamydial urethritis; NSU = non-specific urethritis;

BPVL = mean log₁₀ blood plasma viral loads; SPVL = mean log₁₀ semen plasma viral loads

* figures quoted are number of measurements / number of patients; ** p-value from comparison with controls

Figure 5.1 Plot of \log_{10} semen plasma HIV-1 RNA load versus \log_{10} blood plasma viral load in patients with urethritis and not receiving antiretroviral therapy (study visit 1)

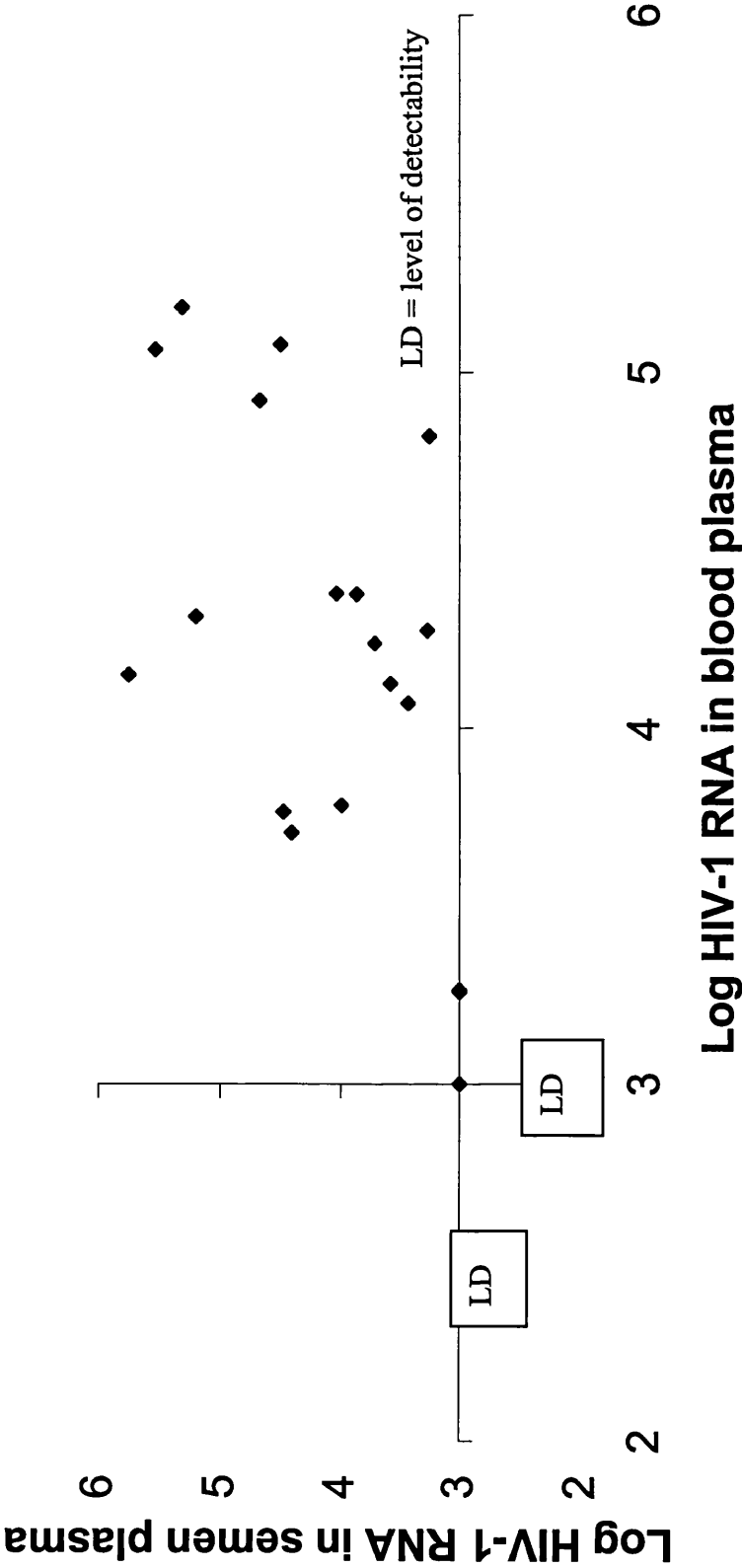


Figure 5.2 Plot of \log_{10} semen plasma HIV-1 RNA load versus \log_{10} blood plasma viral load in patients without urethritis and not receiving antiretroviral therapy (study visit 1)

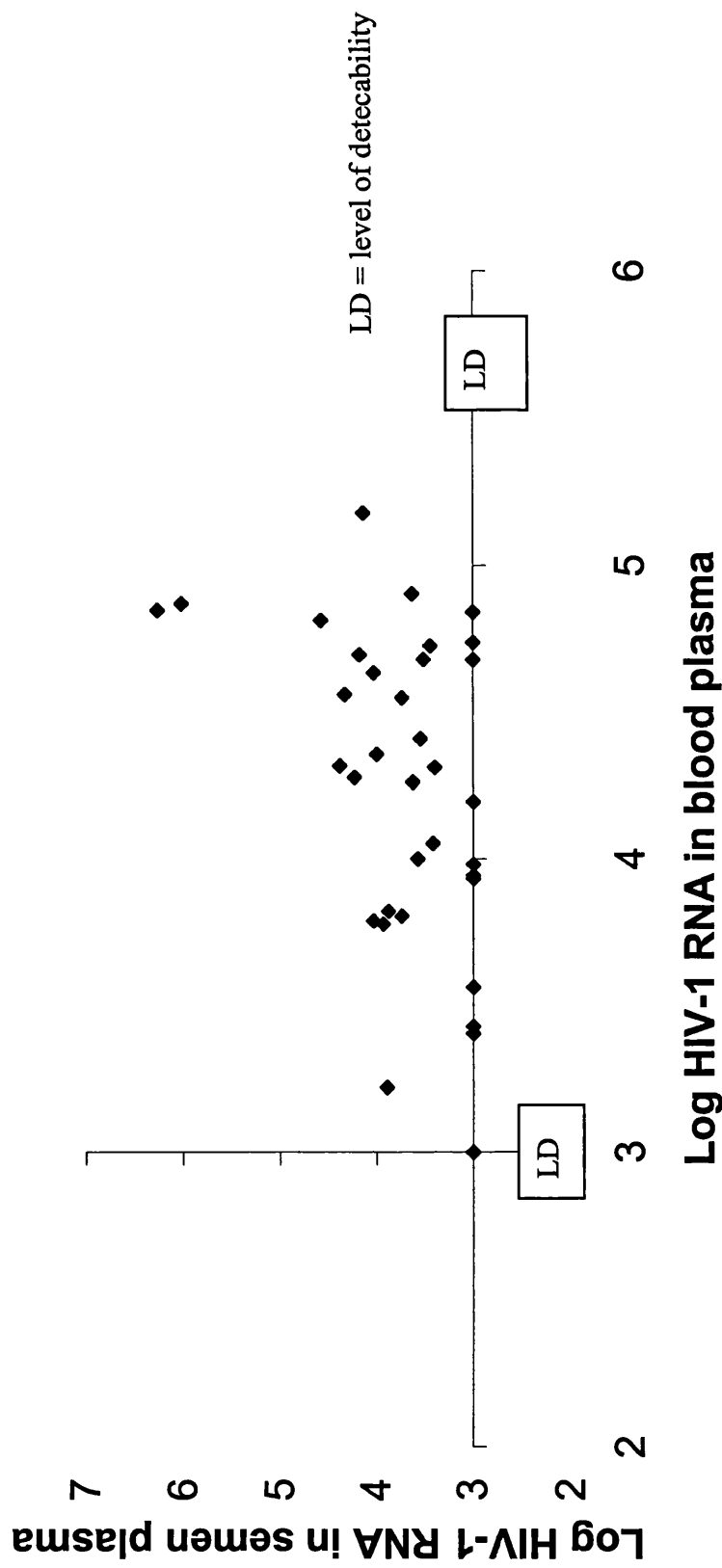
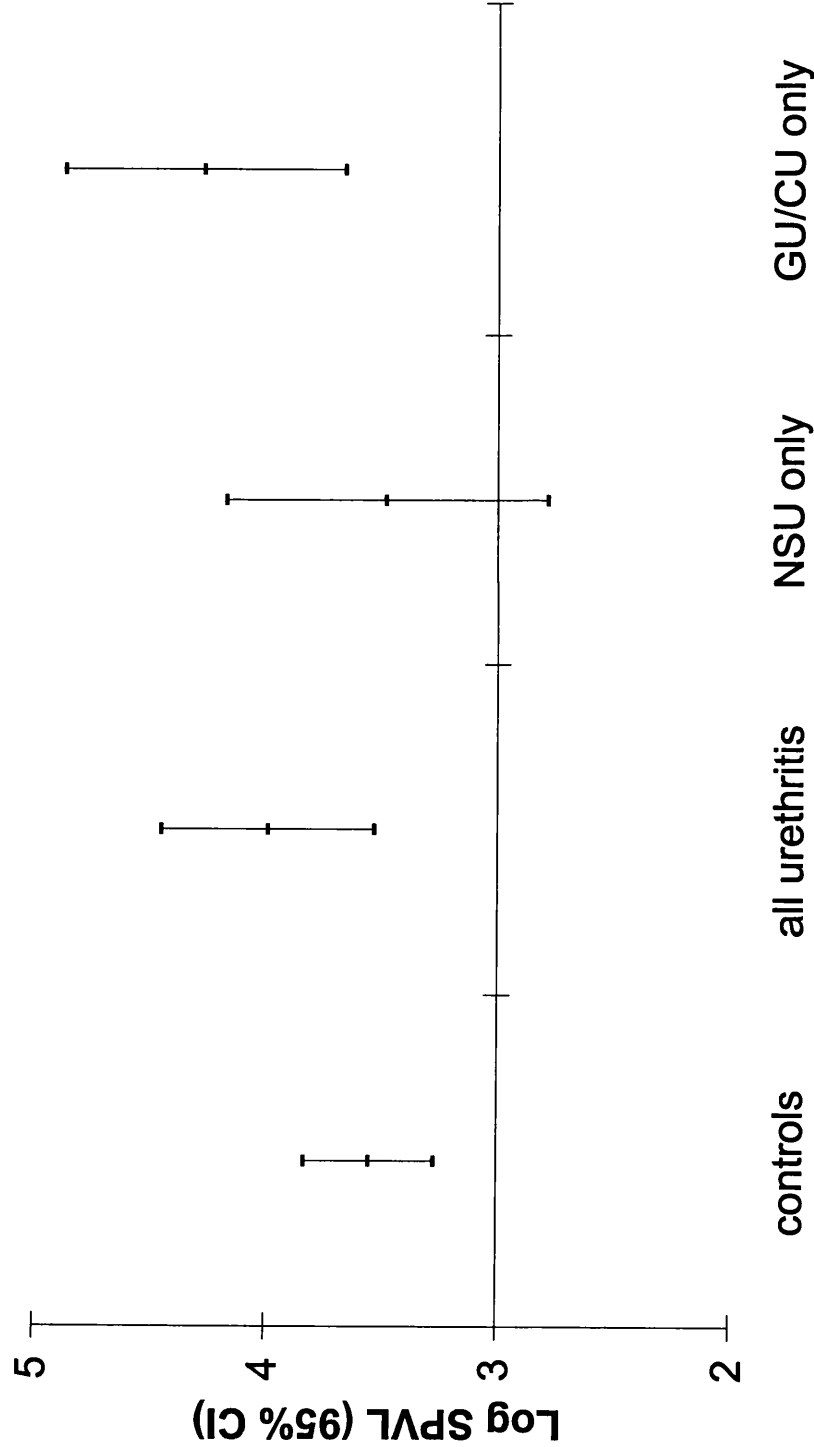
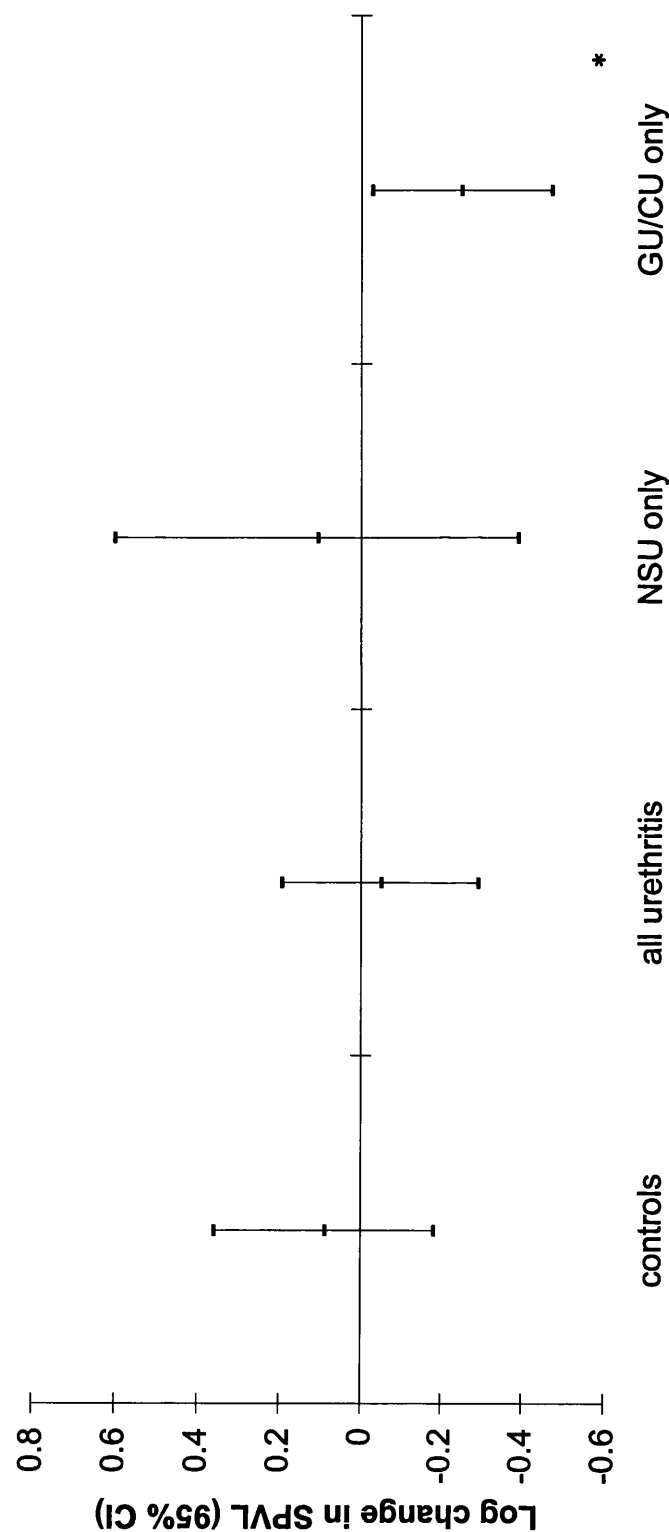


Figure 5.3 Log₁₀ semen plasma viral loads of cases of urethritis and controls at first study visit



GU/CU = gonococcal or chlamydial urethritis; NSU = non-specific urethritis (non-gonococcal and non-chlamydial)
Level of detection = 1000 (3 log), and undetectable viral loads considered as 500 (2.7 log)
P= 0.078 for “all urethritis” vs controls and p=0.014 for “GU/CU only” vs controls.

Figure 5.4 Change in semen plasma viral load from visit 1 to follow-up



GU/CU = gonococcal or chlamydial urethritis
NSU = non-specific urethritis (non-gonococcal and non-chlamydial)
Level of detection = 1000 (3 log₁₀), and undetectable viral loads considered as 500 (2.7 log₁₀). *change = -0.25 log₁₀ (-0.47 - 0.03) p=0.028

CHAPTER 6. THE EFFECTS OF EARLY SYPHILIS ON CD4⁺ T-CELL COUNTS AND HIV-1 RNA VIRAL LOADS IN BLOOD AND SEMEN

6.1 Aims

To examine the effects of early syphilis and its treatment on blood plasma HIV-1 RNA loads (BPVL) and CD4⁺ T-cell counts. To examine, in a prospective sub-study, effects of early syphilis on semen plasma HIV-1 RNA loads (SPVL).

6.2 Methods

6.2.1 Design of retrospective study

All cases of early syphilis in HIV-1 infected patients attending The Mortimer Market Centre between January 2000 and September 2002 were identified from clinic and microbiological records. For each case, controls were selected from among HIV-1 infected men attending with a new episode of gonorrhoea, chlamydia or non-specific urethritis. These sexually transmitted infections (STI) were chosen because of evidence that they do not affect BPVL in men (Cohen et al 1997). Cases and controls with concurrent active genital herpes were excluded because of its effect of increasing BPVL (Mole et al 1997). Controls were matched to cases by date of attendance and combination antiretroviral therapy (CART) status (i.e. on or off CART). Data recorded from patients included age, dates of presentation and HIV diagnosis, ethnicity, antiretroviral history and clinical presentation. All results of syphilis serology, CD4⁺ T-cell counts and routine BPVLs (using the branched-DNA viral load assay; Bayer Diagnostics version 3) taken while patients remained either on or off CART and within 1 year around the presentation of syphilis or STI were also recorded. In those who had presented more than once with a new episode of syphilis

in the period of study, the first diagnosis of syphilis was regarded as the index infection. Controls were only selected and used once.

For every case, a “period of syphilis disease” (POD) was defined (Figure 6.1). A pre-POD and post-POD were also defined as immediately before and after the POD respectively, and equalling its duration. Controls had PODs assigned to them of the same duration as that in their matched case. The POD was intended to reflect a time period, wide enough to include the results of routine BPVLs and CD4 counts but narrow enough to include only times when HIV-1 replication may have been affected by syphilis. The POD included the estimated time that syphilis was incubating (primary = 30 days, secondary = 90 days, early latent = 120 days), the period from the development of symptoms of syphilis to presentation to the clinic, the time from presentation to treatment, and an interval of 2 months post treatment (Figure 6.1). This interval was chosen because of evidence that following treatment of acute systemic infections, such as bacterial pneumonia or malaria, BPVLs may remain elevated for 1 month (Hoffman et al 1999; Sulkowski et al 1998) returning to baseline usually by 3 months (Bush et al 1996; Donovan et al 1996).

As a secondary analysis, an additional period of disease, the “tight-POD”, was defined as a period from 2 weeks prior to two weeks after presentation with syphilis in order to more readily “capture” the effect of syphilis. This shorter disease period was therefore likely to include fewer routine BPVL tests and CD4⁺ T-cell counts.

Approximately 60 cases and 60 controls were required for 80% power if the average change in BPVL from non-POD to POD differed by 0.5 log, relative to a standard deviation of changes in each group of 1, and taking the usual 5% significance level. To increase the power of the study further we attempted to select two controls for every case.

6.2.2 Prospective sub-study

Recruitment to this study took place within the study period of the retrospective study, i.e. from January to August 2002. HIV-1 infected men not on CART for at least 3 months, presenting either with untreated early syphilis (cases) or without syphilis and a negative STI screen on the day of attendance (controls) were recruited. Medical history, blood for syphilis serology, CD4⁺ T-cell counts and BPVL were taken and semen samples provided for assay of SPVL. Semen samples were provided after a routine urethral smear (testing for gonococcal, chlamydial and non-specific urethritis) but before voiding urine. Standard treatment, usually a single dose of benzathine penicillin or a two-week course of doxycycline, was given to those with syphilis. Patients were asked to return at one month, three months and six months after treatment, providing blood and semen samples as previously for HIV-1 RNA load measurement. Additionally, archived residues of BPVL samples routinely taken from participants prior to acquiring syphilis or presentation were retrieved for viral load analysis. 16 cases and 16 controls were required for 80% power if the average change in blood viral loads before and after treatment was 0.5 log compared to controls, relative to a standard deviation of changes within each group of 0.5, and taking the usual 5% significance level. The standard deviation was assumed to be lower than in the main study because the patients were not on CART and therefore possibly more homogeneous. (These differences in sample size calculations between the studies were justified by the results, in as much that cases not on CART were a more homogenous group of patients with respect to viral load range; see table 1). We aimed to recruit 20 cases and 20 controls in case the standard deviation was somewhat higher than assumed.

6.2.3 Testing of Samples (see chapter 2)

Semen and blood samples were centrifuged within two hours of collection and the plasma and cellular components stored at -70°C. HIV-1 RNA was extracted from blood and semen plasma by a silica gel capture method and quantified using a reverse transcribed PCR, clade-B specific assay (Boom et al 1990; Sadiq et al 2002). CD4⁺ T-cell counts were measured using fluorescent activated cell sorter (FACS) analysis. *Treponema pallidum* haemagglutination, rapid plasma reagin (RPR) and fluorescent treponemal assays were used to diagnose or exclude syphilis.

The studies were approved by the Camden and Islington Community Health Services Local Research Ethics Committee.

6.3 Statistical Analysis

6.3.1 BPVLs and CD4⁺ T-cell counts in main study

BPVLs and CD4 counts in cases were compared to those in controls during the pre-POD, POD, and post-POD. Average changes in BPVL and CD4⁺ T-cell count between different disease periods were then estimated separately for cases and controls. For primary analysis, changes were compared between cases and matched controls by testing for an interaction between disease period and whether case or control. All analysis was based on generalised estimating equations (GEE) of STATA 7, because of multiple measurements for patients and matching of controls.

6.3.2 BPVLs and SPVLs in sub-study

BPVLs and SPVLs in cases were compared to those in controls at different study visits. Average changes in HIV-1 RNA loads across study visits were estimated separately for cases and controls, and then these were compared. Statistical analysis was similar to that of the main study. When examining the change in HIV-1 RNA

load, using multiple measurements for patients, then analysis was based on GEE. When comparing HIV-1 RNA loads at particular times (e.g. visit 1), then the t-test was used.

6.3.3 Secondary analysis

Planned subgroup analysis included comparisons of latent with primary/secondary cases. Primary and secondary cases were grouped together because of the frequent difficulty in distinguishing these stages among those with concurrent HIV-1 infection (Rompalo et al 2001; Schofer et al 1996). Also planned for the main study was the comparison between patients on and off CART. To determine whether changes in patient measures differed according to subgroup, the interaction was tested.

6.3.4 Correlation of measures

The correlation between semen and blood viral loads at visit 1 in the prospective sub-study was assessed using the Spearman rank correlation coefficient. In the main study, to assess the correlation GEE was used to regress the changes in viral load on the corresponding changes in CD4⁺ T-cell count.

6.3.5 Outcome definition

For analysis, the log (base 10) of the CD4⁺ T-cell count and viral load were used. Undetectable viral load results were analysed as if half the detectability limit, e.g. 25 copies/ml in the main study.

6.4 Results

6.4.1 Main study

63 cases of early syphilis were identified (15 primary syphilis, 37 secondary syphilis and 11 early latent syphilis) and 104 controls (with gonorrhoea or non-gonococcal infection) were selected. All but two study participants were homosexual men. 27 cases and 39 controls were on CART. The median age, stage of HIV disease and time since diagnosis of HIV were similar for cases and controls (data not shown). Median pre-POD BPVLs and CD4⁺ T-cell counts were similar in cases and controls. Both POD and post-POD BPVLs were not significantly different in cases and controls (Table 6.1).

Overall, changes in BPVLs were not significant when comparing pre-POD to POD or POD to post-POD in all cases, in primary/secondary or early latent cases, in controls or when comparing cases to controls (Figure 6.2). There were no significant associations between change in BPVL and CART status. When using the tight-POD, changes were broadly similar (data not shown).

Overall no differences in CD4⁺ T-cell counts were detected when comparing POD to pre-POD or post-POD in all cases or controls. Significant changes in CD4⁺ T-cell counts through the PODs were detected in early latent cases and these remained significant when compared with changes in matched controls (Figure 6.3). The drop in CD4⁺ T-cell counts in the POD, among early latent cases, was also significantly greater compared to primary and secondary cases, and this remained significant on comparison with matched controls (Figure 6.3). In early latent cases, CD4⁺ T-cell counts decreased relative to controls when moving from pre-POD to POD by 0.104 log (0.020 – 0.189) and then increased relative to controls by a factor of 0.110 log (0.030 – 0.191) following treatment for syphilis. For individuals with baseline CD4⁺ T-cell counts of 500 x 10⁶/ml and 200 x 10⁶/ml, these changes represent drops to 393

$\times 10^6/\text{ml}$ (323 – 477) and $157 \times 10^6/\text{ml}$ (129 – 190) respectively. CD4^+ T-cell counts were significantly lower during the tight-POD when compared with pre-POD (0.072 log; 0.030 – 0.114) and lower by a similar magnitude when compared to the post-POD, but not significantly. The change in CD4^+ T-cell count from POD to post-POD was significantly greater in those on CART (0.081 log) compared to those not (-0.004 log, $p=0.006$; test for interaction). However there was no significant association between CART status and change from pre-POD to POD. The finding of an association between change and CART status did not remain significant when compared to matched controls.

Changes in CD4^+ T-cell count were not significantly related to changes in BPVL among cases or controls (data not shown).

6.4.2 Prospective sub-study

14 cases with early syphilis (2 with primary, 7 with secondary and 5 with early latent) and 20 controls were recruited. All except one case were also included in the main study. During study visits 13 cases and 11 controls had additional blood collected for routine measurement of BPVL by branched-DNA assay as well as the study assay. HIV-1 subtype was determined for all patients where BPVL loads results differed between the two assays by more than 0.5 log. HIV-1 reverse transcriptase and protease genes were sequenced and subtyped by uploading into the Stanford HIV drug resistance database; <http://hivdb.stanford.edu/>). All sequenced viruses were identified as clade-B apart from one from a case of primary syphilis (who acquired HIV in Argentina) who was deemed to have protease subtype K and reverse transcriptase subtype CRF_02_AG. Because of assumed failure of PCR in this patient, his results were not included in subsequent HIV-1 RNA load analysis.

In 9 cases and 9 controls, archived blood plasma samples (see methods) were retrieved. The median time between collection of these samples and first study visit was 96 days (15 – 153) for cases and 120 days (43 –224) for controls ($p=0.59$ for the difference, Mann-Whitney test).

The median time since HIV-1 diagnosis, stage of HIV infection and age at entry into the study were similar in cases and controls (data not shown). All cases had positive syphilis serology with RPR tests ranging from 8 to 512. Prior to the study and before syphilis no differences in BPVLs were detected between cases and controls (Table 6.1). 11 cases and 19 controls gave semen as well as blood samples at the first study visit. 11 cases but only 6 controls were able to give semen samples at at least one follow-up study visit. Overall SPVLs correlated to BPVLs at visit 1 ($r=0.364$; $p=0.048$; Spearman's rank coefficient).

BPVLs and SPVLs were higher among cases compared with controls at the first study visit (Table 6.1). At follow-up visits SPVLs remained significantly higher, and there was some evidence that BPVLs also remained higher, though not significantly. No significant change in mean log BPVL or log SPVL was detected between visits in cases or controls and changes in cases were not significantly different to the changes in controls (Table 6.2).

6.5 Conclusions

Overall the changes in HIV-1 RNA loads across disease periods among cases and relative to controls were not significant in both studies. Overall, CD4 counts were similar in cases and controls in the main study. However, in those with early latent syphilis, when compared with changes in controls, CD4 counts dropped significantly when compared to their pre-POD and then increased following treatment of syphilis.

Table 6.1 Viral loads and CD4⁺ T-cell counts of cases and controls in main and prospective sub-studies

	Syphilis	Controls	P*
Retrospective study			
	N=63	N=104	
Median Pre-POD viral load (range)	5,500 (<50-491,000)	3100 (<50 – 938,300)	0.16
Median POD viral load (range)	16,700 (<50-3,764,500)	1600 (<50 - 6,430,700)	0.13
Median Post-POD viral load (range)	17,400 (<50-696,900)	1400 (<50 – 730,300)	0.10
Median Pre-POD CD4 count (range)	485 (100-1180)	460 (64 – 1140)	0.72
Median POD CD4 count (range)	410 (90-1270)	435 (24 – 1160)	0.79
Median Post-POD CD4 count (range)	475 (120-1400)	440 (20 – 1140)	0.21
Prospective study			
	N=14	N=20	
Median BPVL on available stored samples prior to syphilis	70,359 (2,335 – 184,084)	35,578 (2,947 – 151,652)	0.51
Median BPVL at first study visit	36,638 (16,998-287,030)	20,656 (2,519 - 80,127)	0.01
Median BPVL at study visits 2, 3 and 4	88,786 (17,476 – 299,401)	46,911 (4,845 – 95,973)	0.09
Median SPVL at first study visit	21,060 (<1,000 – 147,531)	3,478 (<1,000 – 24,171)	0.00
Median SPVL at study visits 2, 3 and 4	39,412 (<1000 – 196,840)	2203 (<1000 – 3294)	0.00

POD = Period of syphilis Disease (see methods); Tight POD = stringent period of syphilis disease (2 weeks either side of presentation)

BPVL = Blood plasma viral load; SPVL = Semen plasma viral load

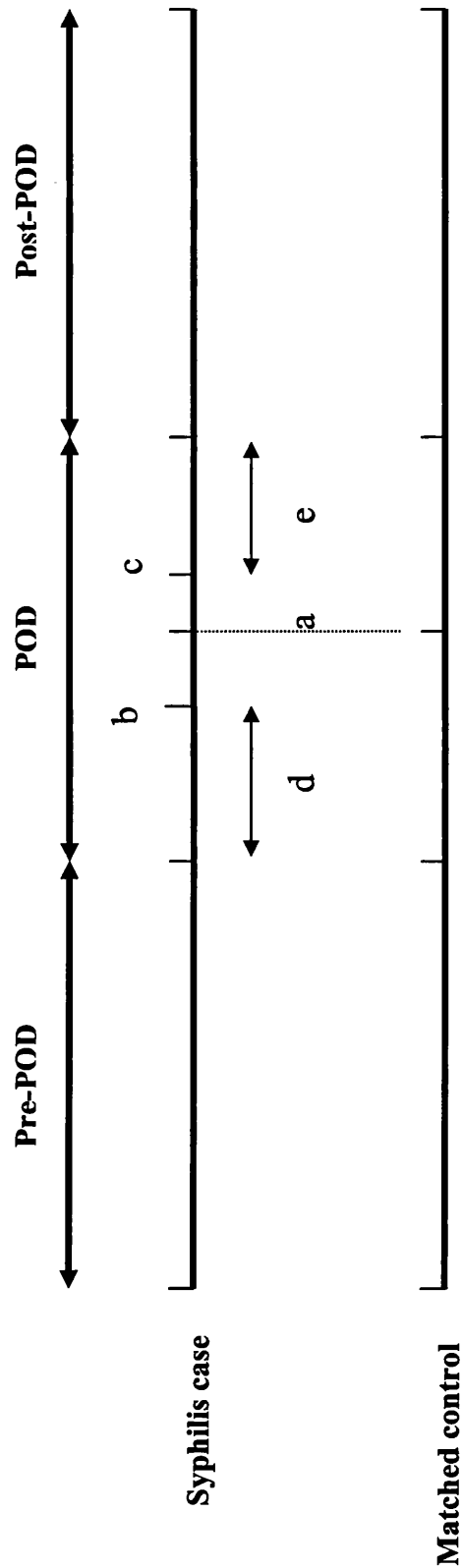
* Statistical tests based on comparison of mean log measures, using GEE where there are multiple measurements per patient, and t-test otherwise

Table 6.2 Changes in log₁₀ blood & semen plasma HIV-1 viral load using RT-QPCR (in-house) viral load assay (Prospective Study)

	Change of log ₁₀ HIV-1 blood plasma viral load ⁺ (95 % CI)	Change of log ₁₀ HIV-1 semen plasma viral load ⁺
From Pre-visit 1 to Visit 1		
Cases	0.035 (-0.312 – 0.382)	N/A *
Controls	-0.206 (-.453 – 0.041)	N/A *
Cases versus Controls	0.241 (-0.172 – 0.655)	N/A *
From visit 1 to post-visit 1		
Cases	0.106 (-0.079 – 0.291)	0.003 (-0.366 – 0.370)
Controls	0.165 (-0.046 – 0.376)	0.003 (-0.298 – 0.305)
Cases versus Controls	-0.066 (-0.338 – 0.206)	-0.027 (-0.369 – 0.422)

*pre-visit 1 semen viral load not available – see study design

Figure 6.1 Schematic diagram representing definition of disease periods of syphilis and matched controls in the Retrospective study



a = presentation to clinic with syphilis (for cases) or STI (for controls)
b = beginning of symptoms/signs (for cases of early latent syphilis b=a)
c = time of treatment of syphilis
d = incubation period of syphilis (primary = 30 days, secondary = 90 days, early latent = 120 days)
e = 2 months post treatment of syphilis
POD = period of syphilis disease
Pre-POD = pre-Period of syphilis disease
Post-POD = post period of syphilis disease

Figure 6.2 Changes in HIV-1 RNA load in main study

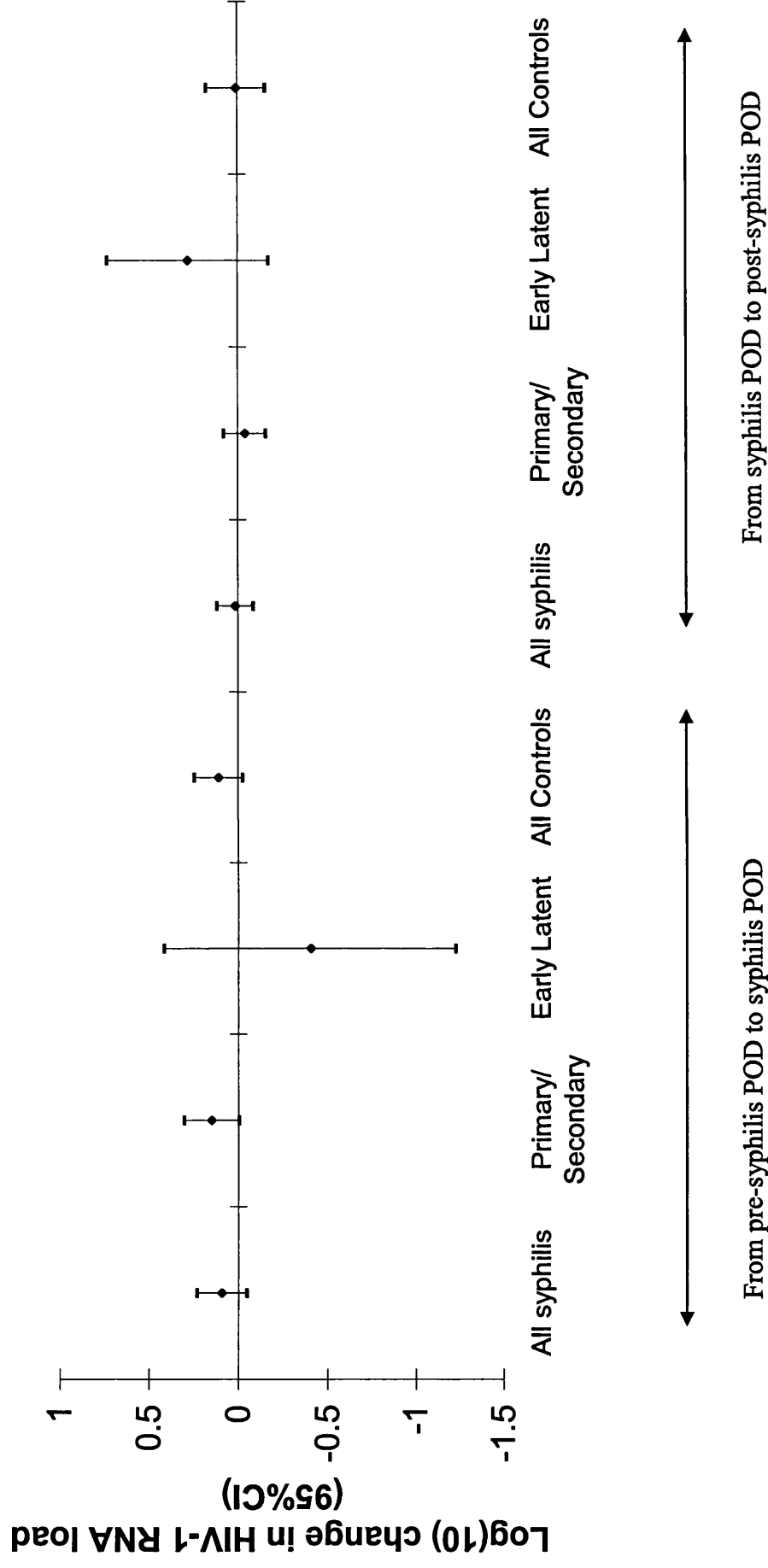
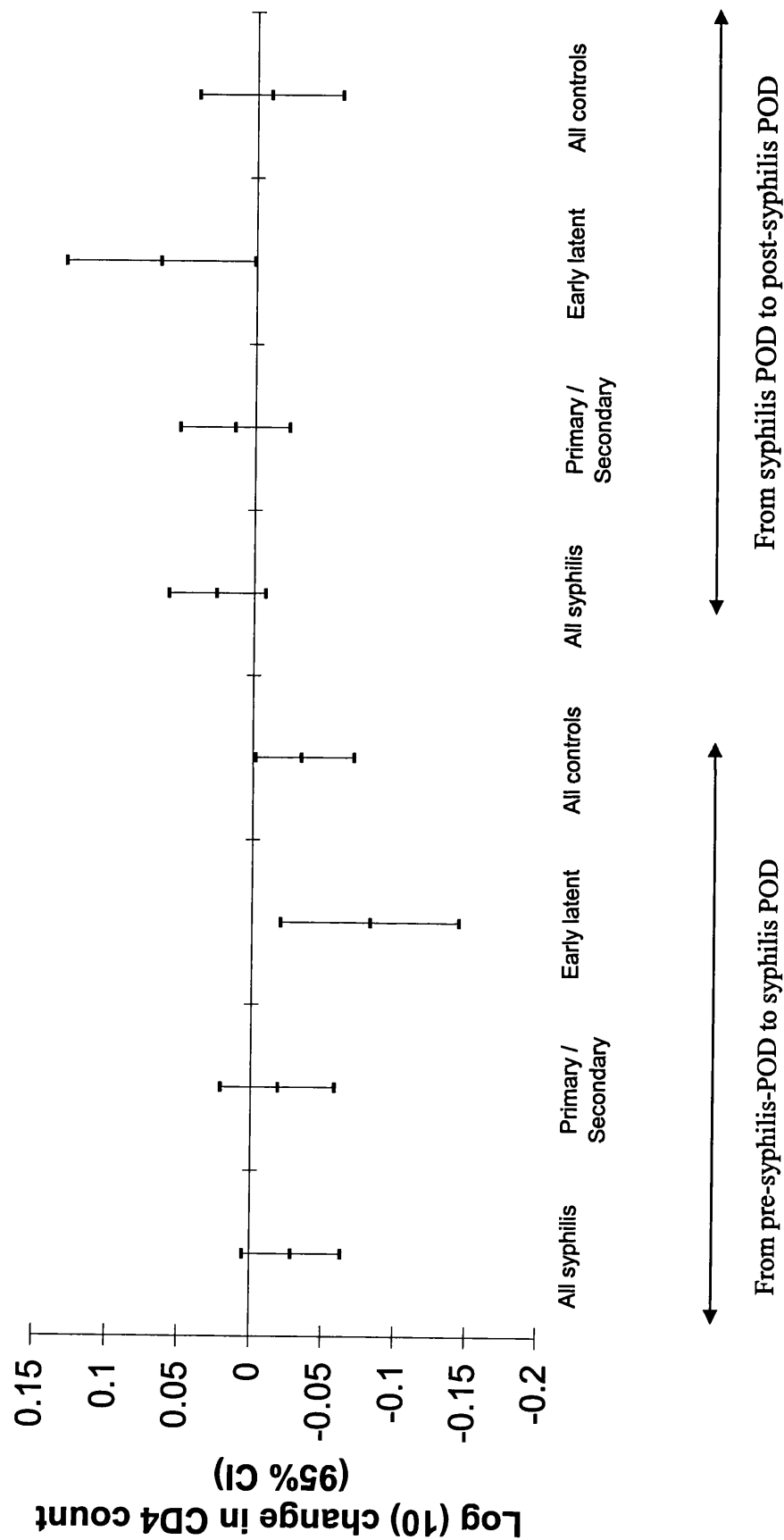


Figure 6.3 Changes in CD4 count in main study



CHAPTER 7. RESEARCH INVOLVING SEMEN DONATION IS FEASIBLE AND ACCEPTABLE AMONG HOMOSEXUAL MEN CO-INFECTED WITH HIV-1 AND GENITAL INFECTIONS

7.1 Aims

To investigate the acceptability and feasibility of research involving semen donation in the context of sexually transmitted infections.

7.2 Methods

Between 1999 and 2002 we conducted two studies into the effects of symptomatic urethritis on HIV-1 RNA loads in seminal plasma in the Bloomsbury Clinic, a large London HIV/STI clinic based at the Mortimer Market Centre (Sadiq et al 2002; Sadiq et al 2004). All patients were homosexual men with HIV infection and were attending for a STI check-up in a nurse-led clinic, either with or without symptoms of urethritis. In the first study, patients were taking combination antiretroviral therapy (CART) whereas in the second, patients were not. During recruitment to the first study, data on the total numbers of patients who were eligible, the total who were offered participation and total who declined were all recorded whereas in the second study these data were not very well recorded. After being offered participation in the studies, eligible patients (including many of those who declined participation) were invited to a face-to-face interview with one of the investigators and answered questions from a structured questionnaire.

The questions in the interview reflected a group of factors that the investigators believed to be potentially important in determining whether patients take part in studies requiring semen donation. These factors were drawn from anecdotal conversations with

patients, staff and informal communications with other investigators involved in semen collection. The factors were: general embarrassment, time constraints, generally not being interested in research (trials), a specific dislike of studies involving the donation of semen, perceived difficulty in producing a semen sample in a public building, perceived difficulty in producing a semen sample because of genital symptoms and discomfort about taking part in studies that looked at how infectious patients were to others. Each patient was asked to grade each factor from 1 to 5 representing low to severe intensity respectively. In addition other data such as age, the most recent CD4⁺ T-cell count, HIV-1 RNA load, stage of HIV-1 disease, antiretroviral therapy status and the presence of symptomatic urethritis were also recorded. Following the interview, clinical samples were collected and the patients were then asked to produce a semen sample. On their first study visit, all patients produced semen samples by masturbation in a designated single unit lavatory within the same building but on a different floor to the clinic. All patients were offered soft pornography for assistance and this was provided when requested as is done for semen donation in fertility clinics.

Among those offered participation in the study, those who agreed to participate were compared with those who declined (overall and by the presence of urethritis) with respect to the most recent CD4⁺ T-cell count and HIV-1 viral load prior to their first study visit and also with regard to their HIV disease stage. For comparisons of age the t-test was used and for CD4⁺ T-cell counts and viral loads the Mann-Whitney test was used. The χ^2 test for trend was used for HIV disease stage. Comparisons of the median scores for factors were also made between those who agreed to take part and those who declined, using the Mann-Whitney test. The odds ratio for agreeing to take part in the

studies, for an increase of 1 unit in each of the factors in the interview, are presented with confidence intervals. For those factors significantly associated with taking part, multiple logistic regression was used to derive odds ratios for each factor adjusting for the others. All analysis was performed using STATA.

7.3 Results

During recruitment to the first study, of 84 eligible patients, 47 (60%) were offered participation and of these, 36 (77%) agreed to take part. Among those with urethritis the acceptance rate was 20/24 (83%) compared to 16/23 (70%) ($p=0.68$) in those without urethritis. During recruitment to the second study, 31 patients agreed to take part. All but three patients who agreed to participate in either study were interviewed and among those who declined participation, all 16 from the first study and 8 from the second study were interviewed.

Overall in both studies, 63 patients who agreed to take part and 24 patients who declined participation were interviewed. 42 of these patients had symptomatic urethritis and 45 did not have urethritis. There were no differences with regard to age, CD4⁺ T-cell count, stage of HIV disease, HIV-1 viral load or antiretroviral therapy status between those who took part and those who declined participation, either overall or comparing those having urethritis or not (data not shown).

As expected, patients who had declined to take part had higher median total scores for the factors investigated. Overall, median scores for embarrassment, time constraints and producing semen in a public building were higher in those who declined to take part than in those who agreed. Among the subset with urethritis, median scores for

embarrassment, and producing semen in a public building were higher among those declining to take part. Among those without urethritis, median scores for time constraints and producing semen in a public building were higher among those declining study participation (data not shown).

The odds ratios of agreeing to take part, for every increase in factor grade by one, were 0.58, 0.6, 0.67, 0.57, 0.66 for embarrassment, time constraints, dislike of semen studies, producing semen in a public building and infectiousness to others respectively ($p < 0.05$; see Table 7.1). In a multiple logistic regression where these factors were controlled for each other only time constraints and producing semen in a public building remained statistically significant (Table 7.1). When examining the odds ratios among those with urethritis, embarrassment and producing semen in a public building were highly significant. When adjusted for each other, only embarrassment remained significant. For every increase in embarrassment grade, the odds of taking part decreased by a factor of 0.30 (95% CI = 0.11 - 0.81) (Table 7.1).

Among those without urethritis, the odds ratios for agreeing to take part for an increase in each factor grade were significant or close to significant for time constraints, dislike of semen studies, producing semen in a public building and infectiousness to others. When adjusted for each other, only time constraints remained significant with an odds ratio of 0.52 (0.29-0.93) for an increase in time constraint grade by one (Table 7.1).

7.4 Conclusions

Research involving semen donation in a busy London HIV/STI clinic was practical and feasible among gay men with HIV infection, even in the presence of genital

symptoms. Time constraints and clearly embarrassment, particularly with regard to producing semen in a public building are important factors in the decision to take part in such studies.

Table 7.1 The odds ratio of taking part in semen studies for an increase in each factor by one unit

Factor	Patient group	Median score (IQR)		Unadjusted		Adjusted*	
		Took part	Declined	Odds ratio	95% confidence intervals	Odds ratio	95% confidence intervals
Embarrassment	Overall	1 (1 – 2)	2.5 (1 – 3.5)	0.58	0.38 – 0.88	0.79	0.46 – 1.34
	Urethritis	1 (1 – 2)	3 (3 – 4)	0.23	0.09 – 0.59	0.30	0.11 – 0.81
	Non-urethritis	2 (1 – 3)	2 (1 – 3)	0.93	0.55 – 1.57	-	-
Time constraints	Overall	2 (1 – 3)	4 (2 – 4.5)	0.60	0.41 – 0.87	0.60	0.39 – 0.91
	Urethritis	3 (1 – 3)	2 (1 – 4)	0.84	0.48 – 1.48	-	-
	Non-urethritis	2 (1 – 3)	4 (3 – 5)	0.46	0.27 – 0.79	0.52	0.29 – 0.93
Not interested in research	Overall	1 (1 – 2)	1 (1 – 3)	0.85	0.52 – 1.38	-	-
	Urethritis	1 (1 – 2)	1 (1 – 2)	0.98	0.47 – 2.03	-	-
	Non-urethritis	1 (1 – 2)	1 (1 – 3)	0.72	0.35 – 1.48	-	-
Studies involving semen donation	Overall	1 (1 – 2)	1 (1 – 4)	0.67	0.47 – 0.96	0.89	0.57 – 1.39
	Urethritis	1 (1 – 1)	1 (1 – 2)	0.78	0.42 – 1.44	-	-
	Non-urethritis	1 (1 – 2)	1.5 (1 – 4)	0.63	0.40 – 1.01	0.74	0.40 – 1.36
Semen donation in public building	Overall	2 (1 – 3)	4.5 (2 – 5)	0.57	0.40 – 0.81	0.64	0.42 – 0.98
	Urethritis	2 (1 – 3)	4 (3 – 5)	0.34	0.16 – 0.74	0.47	0.20 – 1.09
	Non-urethritis	2 (1 – 3)	5 (1 – 5)	0.70	0.47 – 1.05	0.84	0.50 – 1.41
Presence of genital symptoms	Overall	1 (1 – 3)	2 (1 – 4)	0.83	0.60 – 1.15	-	-
	Urethritis	1 (1 – 3)	3 (2 – 4)	0.64	0.37 – 1.11	-	-
	Non-urethritis	1 (1 – 3)	1 (1 – 3)	0.92	0.60 – 1.42	-	-
Studies examining infectiousness for HIV	Overall	1 (1 – 1)	1 (1 – 2.5)	0.66	0.43 – 1.01	0.72	0.44 – 1.18
	Urethritis	1 (1 – 1)	1 (1 – 1)	0.80	0.39 – 1.62	-	-
	Non-urethritis	1 (1 – 1)	1 (1 – 3)	0.60	0.34 – 1.07	0.69	0.36 – 1.31

Legend for Table 7.1

* Factors examined were:-

Overall: Embarrassment, Time constraints, Dislike Semen studies, Producing semen in Public building, Infectiousness to others.

Urethritis: Embarrassment, Producing semen in Public building.

Non-Urethritis: Time constraints, Dislike Semen studies, Producing semen in Public building, Infectiousness to others.

IQR = inter quartile rang

CHAPTER 8. DISCUSSION

This thesis has focussed primarily on the levels of HIV-1 measured in semen and blood of homosexual men during sexually transmitted infections, namely urethritis (due to gonorrhoea, chlamydia or NSU) and early syphilis. It has examined effects of these infections in patients receiving or not receiving antiretroviral therapy. In addition, in those with early syphilis the effects on CD4⁺ T-cell counts were also analysed.

8.1 Effects of urethritis on SPVL in men receiving CART

In this study, in 18 of 24 cases of urethritis and 13 of 16 controls, SPVL was undetectable and where tested HIV-1 proviral DNA was also largely undetectable. In general there was a strong association between detectability of HIV-1 RNA in seminal plasma with that in blood plasma in all patients confirming previous findings in patients taking antiretroviral therapy, who do not have urethritis (Liuzzi et al 1999; Vernazza et al 1997a; Vernazza et al 1997b). This study therefore provided important evidence that antiretroviral therapy may not only reduce sexual transmission of HIV-1 by reducing viral load in semen but may also reduce the potential facilitating effects of sexually transmitted infections on HIV-1 transmission.

Among many patients in this study (both cases and controls) HIV-1 RNA was suppressed in the semen despite them being on protease inhibitors or non-nucleosides associated with poor penetration into the genital tract (see section 1.5.7). Perhaps the high concentrations of nucleoside analogues in semen in these patients were adequate to suppress viral load. This may have implications for treatment strategies designed to reduce toxicities by using nucleoside analogue sparing regimens. It is biologically

plausible that in the absence of nucleoside analogues low concentrations of drug in semen may result in failure of HIV-1 suppression in the genital tract and subsequent overall failure of the regimen. An important study has demonstrated that in patients with undetectable plasma viral load (<50 copies/ml) resistance mutations to protease inhibitors and nucleoside analogues are found in seminal plasma (Solas et al 2003). In one patient in this study resistance mutations associated with a previous protease inhibitor regimen, at least six months previously, were present in seminal plasma, suggesting persistence of resistant virus in semen. This study has not commented on whether virological failure was observed in these patients subsequently in plasma.

SPVL was detectable in 3 of 16 controls (at low levels) and in 6 of 24 cases. In three of the cases SPVLs were reduced following treatment for urethritis. In one of these cases, with poorly controlled BPVL the reduction in SPVL was reduced twenty fold following treatment for gonorrhoea. These observations suggest that, at least in some patients, antiretroviral therapy may not always control the potentiating effects of urethritis on HIV replication in the genital tract.

There was a suggestion that among those with detectable BPVL, SPVL was more likely to be detected in cases compared with controls, but we believe this was explained by the higher BPVLs in cases (see Tables 3.2 and 3.3).

This study also confirms that in patients whose BPVLs may be undetectable for more than 1 year and whose SPVLs are also undetectable, HIV-1 provirus may still be detectable in semen (Liuzzi et al 1999; Tachet et al 1999; Zhang et al 1998) as well as in blood (Demeter et al 2002).

In the three cases (19-21), in whom antiretroviral therapy had clearly failed virologically prior to acquiring urethritis, SPVLs were lower than BPVLs and did not change significantly following urethritis treatment. These results may seem surprising

given that urethritis might have been expected to raise SPVL above BPVL in the presence of failing therapy. There are several possible explanations for this observation. Firstly, even though BPVLs and SPVLs were high in these patients it is possible that antiretroviral therapy may still have been sufficiently effective to contain the potentiating effects of urethritis on SPVL. Alternatively it is possible that the viral load in the semen of these patients was already too high for there to be a potentiating effect of urethritis on replication. Thirdly, in a previous study showing large increases in SPVL during urethritis, patients were not asked to void urine before ejaculating (Cohen et al 1997) (personal communication, Dr Irving Hoffman, AIDSCAP Malawi Research Group). Previous studies on vasectomised patients have demonstrated that the vast majority of HIV-1 in semen arises distal to the vas deferens (Anderson et al 1991; Krieger et al 1998), but the source of increased SPVL following urethritis has not been established. HIV-1 contained in the urethral discharge may be an important part of this source. In patients 20 and 21, urine was voided just prior to producing semen samples and HIV-1 provirus was found in first voided urinary cells of patient 20. Thus these patients may have had increased amounts of HIV-1 RNA in the urethra that was washed away after voiding urine, before semen collection. However patient 19 voided urine after semen production and still had no increase in SPVL. Other explanations for rises in SPVL not being seen in these patients include decreased replicative fitness of HIV-1 due to the presence of drug resistant mutations prior to acquiring urethritis or alternatively HIV-1 acquiring drug resistance mutations during urethritis resulting in SPVLs not falling following treatment. Finally it is possible that rises in SPVL associated with urethritis, previously observed in Africa, do not happen to the same degree in the developed world. However, in this thesis we have demonstrated that increases do occur (see chapter 5).

Patients such as these may have increased infectiousness for drug-resistant strains of HIV-1 and contribute to the prevalence of sexually transmitted drug resistant HIV-1 (Brodine et al 1999; Erice et al 1993; UK Collaborative Group on Monitoring the Transmission of HIV Drug Resistance. 2001)

The study has shown that antiretroviral therapy may be important in limiting the effect of sexually transmitted infections on the transmission of HIV-1. For this potential benefit to be fully realised viral load in semen as well as in peripheral blood plasma may need to be optimally suppressed. Where this is not the case urethritis may still increase the risk of HIV-1 transmission of both wild-type and drug-resistant strains.

8.2 Drug-resistant strains of HIV-1 in the semen of men with urethritis

There is increasing concern about the sexual transmission of drug resistant HIV-1, especially in the context of the continuing growth of the epidemic. Previous reports of drug resistant virus in genital secretions (Byrn et al 1998; Eron et al 1998; Eyre et al 2000; Kroodsma et al 1994; Mayer et al 1999) had not investigated the potential for STIs to augment the transmission of resistant virus. The role of acute STIs in this phenomenon is potentially important as epidemiological and biological data already suggest that STIs may enhance wild-type HIV-1 transmission and, additionally the presence of STIs serve as a marker for those continuing to engage in sexual activity capable of transmitting HIV-1. We genotyped the virus in blood and semen of four of our patients on failing antiretroviral regimes and concurrent urethritis and found drug resistant virus in both compartments in all cases. In one of these patients, drug resistance in semen and blood was associated with a seminal plasma viral load that was 20-fold higher during gonorrhoea than following its

treatment. These data do suggest that in some individuals without maximally suppressed blood plasma viral load, the combination of STI's and ART may actually promote the transmission of drug resistant virus.

Our data contribute to growing concern about ongoing HIV spread and transmission of drug resistant virus. The prevalence of transmitted resistance has grown in recent years (Grant et al 2002; Little et al 2002; UK Collaborative Group on Monitoring the Transmission of HIV Drug Resistance. 2001). It is also evident that HIV-infected men are often co-infected with other STIs (Dukers et al 2002), which increase SPVL (Cohen et al 1997) and as we now show may lead to the presence of resistant virus in semen. Ongoing unsafe sexual activity and STIs in the era of ART may lead to groups of individuals driving the spread of wild-type as well as drug resistant HIV-1. Therefore the sexual health of HIV-infected individuals must remain of primary concern.

Unfortunately, the low viral load and small semen sample volume in many cases and controls precluded a formal comparison of the prevalence of resistant virus in semen between these two groups and larger studies are required in order to confirm our preliminary results. In conclusion our findings suggest that STIs may enhance the transmission of drug-resistant HIV-1 from men receiving suboptimal ART.

8.3 Effects of urethritis on SPVL in homosexual men not receiving CART

This study of 55 homosexual men is the largest as yet from the developed world examining effects of STIs on SPVL in those not on antiretroviral therapy. Compared with controls without STIs, SPVLs were approximately 5-fold higher in those with GU or CU but were not higher in those with NSU. Additionally, SPVLs were similar to BPVLs among those with GU or CU whereas SPVLs were

approximately half a log lower than BPVLs among controls and those with NSU. Treatment of GU or CU resulted in reduction in SPVLs by a small but significant amount over one to two weeks. Thus these results indicate GU and CU, though not NSU, increase SPVL in those not on ART.

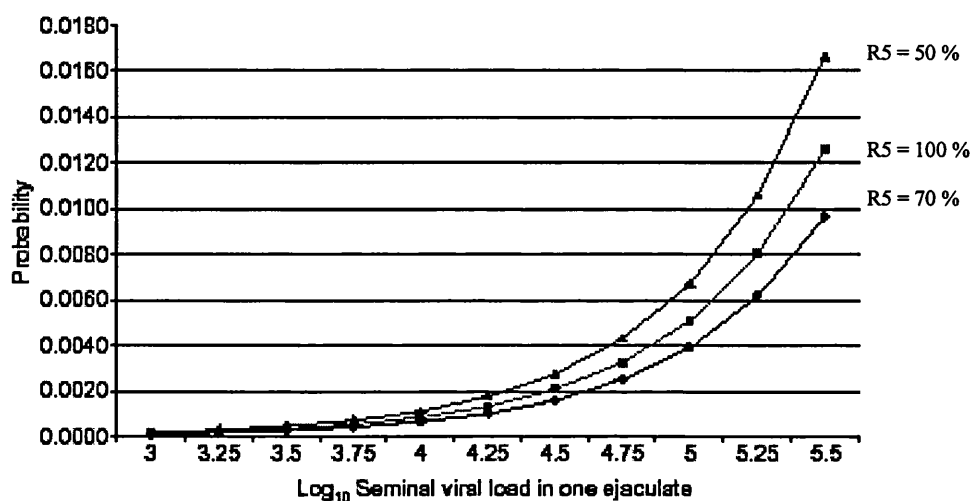
Previously, similar studies in the developed world have been small and few in number. A case report of a 2-log reduction in SPVL following treatment of CU (Eron, Jr. et al 1996) did not comment on changes in BPVL and in a study of 4 patients not on ART with asymptomatic urethritis, two had high SPVLs but with BPVLs that were also high (Winter et al 1999). Another study from the UK, showed small but statistically significant decreases in semen HIV-1 proviral load following treatment of three cases of GU and one case of symptomatic NGU (Atkins et al 1996). These changes in proviral load might be expected given the marked, cellular inflammatory response observed in GU and the association between detectability of cell associated virus and semen leukocyte count (Xu et al 1997). However, increases in both cell-free and cell-associated HIV-1 in semen are important as both forms of virus may be transmissible (Zhu et al 1996). In the un-inflamed genital tract, though detection of proviral and cell free HIV-1 in semen are associated (Ball et al 1999a), cell-free HIV-1 RNA appears phylogenetically distinct from cell associated HIV-1 (Paranjpe et al 2002). Previous work has suggested that cell-free virus in semen is derived locally in the genital tract during urethritis (Ping et al 2000) but it remains unclear whether the increase in HIV-1 RNA in semen during STIs is derived from seminal leukocytes.

In sub-Saharan Africa, urethritis has been associated with increased genital shedding of HIV-1, with median differences of over 100,000 copies/ml in SPVL observed in GU cases compared to those without STIs, an approximately 5-fold difference (Cohen et al 1997; Moss et al 1995). This relative effect of GU/CU on

SPVLs are similar to those in our study, however the absolute effect on SPVLs are considerably higher than our study, where the difference in median SPVL between those with GU and controls was only 15,000 copies/ml. Explanations for the observed differences between the two settings include patients in the African study being more likely to have late stage HIV disease at presentation (baseline CD4⁺ T-cell counts appeared slightly higher in our study), the higher baseline viral loads in blood and semen previously observed in Africa when compared with the developed world (matched for CD4⁺ T-cell count (Dyer et al 1998b)) and the heightened states of immune activation observed in Africa appear to be environmentally driven (Clerici et al 2000). BPVLs at baseline were higher by up to 1 log in the African study compared with our study.

A probabilistic model of HIV-1 transmission between heterosexuals has been developed from biological and epidemiological data from the United States and Switzerland (Chakraborty et al 2001; Figure 8.1). A model such as this is unlikely to be completely accurate for homosexual or African men or for the effect of STIs on SPVL. However, crudely applying this model to our data suggests that the HIV male-to-female per contact transmission probability would increase 3-fold from approximately one per thousand to up to three per thousand during GU or CU. If this model is applied to the African data, one would expect to see an increase of transmission probability from three per thousand to nine per thousand. It is possible therefore that the effects of these STIs on SPVL may not have as great an impact on transmission risk of HIV-1 in the developed world as in Africa. Clearly, however more appropriate models and further research on the implications of our findings on HIV-1 transmission are required.

Figure 8.1 Model relating infectivity to semen plasma viral. Male-to-female per-sexual contact HIV transmission probability for different R5 virus counts (for 50% of target co-receptors being CCR5). The horizontal axis represents \log_{10} seminal viral load in one ejaculate and the vertical axis represents the male-to-female per-sexual contact HIV-1 transmission probability. Three different lines represent different R5 virus counts, 50%, 70% and 100% (adapted from Chakraborty et al 2001).



Our work suggests that in the small number of patients with chlamydial infection, the effect on SPVL appeared to be just as pronounced as those with gonorrhoea. This is important as *Chlamydia trachomatis* is a common cause of urethritis in homosexual men (Benn P et al 2000; Varela et al 2003). Our findings with NSU may not be surprising given that infection may not always cause this condition. Furthermore, the diagnosis of NSU by microscopy is subject to considerable observer variation (Smith R et al 2003) as opposed to the microbiological diagnoses of gonorrhoea and chlamydia by culture or by nucleic acid amplification tests. However we did restrict NSU cases to those who were asymptomatic and had higher polymorph counts of 10 p/hpf or more. Additionally our findings in relation to NSU may not apply to heterosexual men as its aetiology maybe

different to those in homosexual men. For example, *Trichomonas vaginalis*, an important cause of urethritis in heterosexuals in some settings (Hobbs et al 1999; Wendel et al 2003) and associated with increased shedding of HIV-1 in semen (Hobbs et al 1999), is rare in homosexuals (Varela et al 2003). Further research with more patients might more rigorously address the issue of how the magnitude of changes in HIV-1 viral load differ between cases of NSU, GU, and CU.

In the previous study (chapter 3) we demonstrated that in a group of men similar to those of this study but receiving fully suppressive ART and with GU or CU, SPVLs remained undetectable (Sadiq et al 2002). In a small sub-set of patients in whom virus was not suppressed in blood, high amounts of drug resistant virus were detected in seminal plasma, though in only one case did treatment of gonorrhoea result in reduction of SPVL (Sadiq et al 2002; Taylor et al 2003b). This study, in men not receiving CART, would thus strengthen the notion that antiviral therapy attenuates effects of STIs on genital shedding of HIV-1. As CART becomes more widely used, these attenuating effects, need to be confirmed in developing world settings because of high rates of STIs there and potential for widespread transmission of drug resistant HIV-1.

This study has demonstrated that gonococcal and chlamydial urethritis among homosexual men in the UK increases shedding of HIV-1 in semen and treatment of urethritis reduces its shedding. Controlling STIs in HIV-1 infected homosexual men may be critical in controlling the spread of HIV-1 among them.

8.4 Effects of early syphilis on CD4⁺ T-cell counts, BPVL and SPVL

We studied the effects of early syphilis and its treatment on HIV-1 RNA loads in blood and on CD4⁺ T-cell counts in a retrospective case control study and on HIV-1

RNA loads in semen in a small prospective sub-study. BPVLs were similar in cases and controls in the retrospective study but both BPVLs and SPVLs were higher in cases compared with controls in the prospective study. Overall however, the changes in HIV-1 RNA loads across disease periods among cases and relative to controls were not significant in both studies.

Primary and secondary syphilis are associated with marked immune activation (Engelkens et al 1993; McBroom et al 1999; Radolf et al 1991; Radolf et al 1995; Tosca et al 1988; Van Voorhis et al 1996) and patterns of cytokine changes seen in early syphilis have been associated with increased HIV-1 replication via NF-kappa B mediated pathways (Norgard et al 1996; Theus et al 1998). *Treponema pallidum* and early syphilis are associated with up-regulation of the chemokine co-receptor CCR5 on lymphocytes and macrophages (Sellati et al 2000) and increased expression of these co-receptors is associated with increased viral replication and disease progression in HIV infection (Reynes et al 2000; Reynes et al 2001). Despite this, our studies failed to detect HIV-1 viral load increases in syphilis cases. Complex immunological responses to syphilis may partly explain these findings. For example, TNF- α , interleukin-10, IL-2, and γ -interferon are associated with syphilis (Radolf et al 1991) or found in syphilitic lesions (Van Voorhis et al 1996). TNF- α is associated with enhanced HIV-1 replication in lymphocytes, but with its suppression in macrophages (Kornbluth et al 1989) and IL-10 produced in abundance during early syphilis is associated with inhibition of production of TNF- α , IFN- γ and IL-2 (Herbein et al 1997). *Treponema pallidum* is localised in lymphoid tissue during early syphilis (Yobs et al 1968), and it is possible that increased viral replication occurring in lymphoid tissues (Embretson et al 1993; Pantaleo et al 1993a) was not reflected in changes in BPVL.

Higher SPVLs observed in cases in the prospective study probably reflected the higher BPVLs of this group of patients. SPVLs did not decrease significantly following treatment of early syphilis perhaps implying that syphilis itself had little effect on SPVLs. Thus the hypothesis that semen is a source of increased HIV-1 infectivity during early syphilis is not strongly supported by these data.

Syphilitic ulcers may be an important source of HIV-1 as in the case of genital herpes (Schacker et al 1998a). Syphilitic ulcers are infiltrated by CD4⁺ T-cells, histiocytes and CD8⁺ cytotoxic T-lymphocytes thus providing a mechanism for transmission of both cell free and cell associated HIV-1 (Engelkens et al 1993; McBroom et al 1999; Tosca et al 1988). Clearly however this remains an area for further research.

Overall, CD4⁺ T-cell counts were similar in cases and controls in the main study. However, in those with early latent syphilis, when compared with changes in controls, CD4⁺ T-cell counts dropped significantly when compared to their pre-POD (Period of disease) and then increased following treatment of syphilis.

As the immune response to syphilis involves recruitment of cytotoxic T-lymphocytes and macrophages to sites of primary and secondary syphilitic lesions (McBroom et al 1999; Van Voorhis et al 1996), CD4⁺ T-cell count changes may be explained by diversion of CD4⁺ cells from blood to other tissues, particularly lymphoid and skin. Also, marked immune activation may have resulted in increased apoptosis of CD4⁺ T-lymphocytes, independent of any effect on viral replication (Gougeon et al 1996). In addition, the duration of untreated syphilis is longer in those who present with early latent syphilis compared with those who present with primary or secondary syphilis and this may have been important in producing the effect on CD4 count. It is less likely that CD4⁺ T-cell decline was due to cell-lysis associated

with increased HIV-1 replication, as CD4⁺ T-cell count changes were not related to changes in BPVLs. Active syphilis has previously been associated with a five percent reduction in CD4⁺ T-cell percentage in both HIV-2 infected and uninfected patients respectively (N'Gom et al 1997). Thus, the CD4⁺ T-cell count changes we observed may simply reflect the effects of syphilis, acting independently of HIV-1 infection. Whether the reversible drop in CD4⁺ T-cell counts continues into the late latent stage of syphilis remains an area for further research. Finally, if these CD4⁺ T-cell count changes were to translate into HIV-1 disease outcomes, widespread screening and treatment programmes for syphilis in HIV-1 infected patients at risk become even more important to establish.

Our studies were not powered to detect small changes in viral load during syphilis infection. In the main study it is possible that CART may have limited the potential changes in BPVL through PODs, though in our subgroup analyses we found no significant evidence of differential change by CART status. Although we only recruited 14 of the 20 cases intended in the prospective study, the 95% confidence intervals for changes after treatment for syphilis relative to controls were comparatively small ranging from -0.34 log to 0.21 log and -0.37 log to 0.42 log for blood and semen viral loads respectively. Controls were also selected differently for the main and sub-studies, and the choice of controls with STIs in the main study may have underestimated viral load effects because of a potential effect of these STIs on viral load. However, although previous work has shown that genital gonococcal infection can increase BPVL and reduce CD4⁺ T-cell counts in female commercial sex-workers in Africa (Anzala et al 2000), other work from Africa (Cohen et al 1997) and the studies in this thesis from our clinic have shown that it does not increase BPVL (chapter 5) or reduce CD4⁺ T-cell count (data not shown). Another potential

limitation of the study is that POD definitions may have resulted in a dilution of the effect of syphilis. In the UK, early latent syphilis is diagnosed in those with positive syphilis serology combined with either a negative test for syphilis in the previous two years or a clinical picture compatible with infection with syphilis in that time. However patients with concealed primary chancres or those presenting between the stages of primary and secondary disease may also erroneously be diagnosed with early latent syphilis. The choice of the incubation period of 120 days for early latent syphilis was a pragmatic one. Thus in some of those with early latent syphilis, the incubation periods used may have been excessively short or long resulting in considerable overlap of syphilis disease state in pre-POD and POD. This would have resulted in reduced changes in BPVL and an underestimation of the effect on CD4⁺ T-cell count. In those diagnosed with primary or secondary syphilis, the duration of PODs may have been too wide, again resulting in an underestimation of effect. However the fact that no changes in viral load were seen when comparing tight-POD to post-POD does not support this explanation.

Our data demonstrate that early syphilis has little, if any, effect on blood or semen plasma HIV-1 RNA loads. Early syphilis, particularly early latent syphilis is associated with lower CD4⁺ T-cell counts, probably independently of HIV infection. Treatment of early latent syphilis results in recovery of CD4⁺ T-cell count. If early syphilis infection increases the risk of HIV transmission from men, then this study suggests that this is unlikely to be due to an increase in blood or semen plasma levels and therefore more likely due to increased shedding from associated genital skin lesions as has been seen with genital herpes.

8.5 Feasibility study

Studies on the feasibility of semen donation have largely been done in relation to recruitment to fertility clinics. Though many studies have been published looking at semen in relation to STIs and HIV, there is no data on the feasibility of such studies and most of these studies in the developed world have been small. It is possible that such research may be hindered by investigator beliefs that large studies are not feasible. In addition the recruitment of select groups of patients more willing to produce semen samples for research may result in unrepresentative study groups.

We have demonstrated good recruitment to two studies looking at the effects of symptomatic urethritis on HIV-1 RNA load in the semen of gay men. We have also demonstrated that patients recruited were largely representative: there appeared to be no differences in age, stage of HIV disease, CD4⁺ T-cell count, HIV-1 RNA load and antiretroviral therapy status between patients who agreed or declined to take part.

Time constraints are clearly an important factor in the decision to take part in such studies. Why this was only apparent in those without urethritis in our study is unclear, and might possibly be due to chance. It is possible that the men presenting with urethritis differed from those without with regard to social and employment factors. Also, perhaps the extra time and effort required to manage these patients influenced their decision to take part.

Embarrassment, particularly with regard to producing semen in a public building appeared to be more important in those with urethritis. Though patients were seen initially in a nurse-led STI clinic, it was housed within a dedicated HIV clinic, where patients knew staff and doctors. It is possible that the embarrassment felt by patients simply reflected having an STI rather than taking part in a semen study.

Importantly, genital symptoms did not appear to be an important factor for non-recruitment.

It may be that if patients were allowed to produce semen samples at home, participation in studies such as this might be greater. However this was not practically possible for those with urethritis as a semen sample was required prior to treatment of their urethritis at baseline when they were interviewed. So the questionnaire data relates to this baseline point. As part of the protocol of both studies, patients were allowed to produce semen samples for their second or third study visits at home, provided they brought the samples to the clinic within two hours and the majority of the patients took this option. However the compliance with producing specimens in the clinic was very good as 36 of 47 eligible patients (77%) in the first study donated baseline semen specimens in a clinic environment.

This study has shown that research involving semen donation in a busy London HIV/STI clinic was practical and feasible among gay men with HIV infection, even in the presence of genital symptoms. Allowing patients to produce semen samples at a time convenient for them or at home may further enhance recruitment to such studies.

8.6 Overall conclusions

Overall the studies demonstrate that gonococcal and chlamydial urethritis, but not NSU, increase SPVL and that these effects are attenuated almost completely in those on suppressive antiretroviral therapy. In comparison with the increases previously demonstrated in Africa, the relative effects on those not on therapy were similar but the absolute increases were smaller. In those on poorly suppressive antiretroviral regimes SPVL may be high in those with gonococcal infection and

multidrug resistant HIV-1 may be found in both genital and systemic compartments. The studies also demonstrate very little effect of early syphilis on either blood or semen plasma viral load but demonstrate an apparent drop in CD4⁺ T-cell count, particularly in those with early latent syphilis that is reversible on treatment of syphilis. In a study on the feasibility of studies involving semen donation, it was shown that such studies, important for studying transmission of HIV-1 were quite acceptable, even among men with genital symptoms.

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APPENDIX 1. PHOTOGRAPHIC PLATES

All photographs are printed with signed consent from patients in the study described in chapter 6.

Plate 1

Maculo-papular rash associated with secondary syphilis in a study patient with HIV-1 infection



Plates 2 and 3. Palmar /plantar lesions of secondary syphilis in a study patient with HIV-1 infection.



APPENDIX 2. REAGENTS FOR IN-HOUSE RT-QPCR ASSAY

Buffers

RNA extraction lysis buffer

100ml 0.1M Tris/HCl, pH 6.4

+ 120g guanidine thiocyanate

+ 22ml 0.2M EDTA, pH 8.0

+ 2.6g Triton X-100

Aliquot in 25ml quantities and store at 4°C

RNA extraction wash buffer

100ml 0.1M Tris/HCl, pH 6.4

+ 120g guanidine thiocyanate

Aliquot in 26ml quantities and store at 4°C

RNA elution buffer

Promega nuclease free water (cat. No. P1194)

Aliquot in 650µl aliquots using a filter tip

Store at 4°C

Chemiluminescence Buffer A

Casein blocking buffer (Genosys)	12.5ml
----------------------------------	--------

Tween 20	1.25ml
----------	--------

Sodium azide (10% stock soln.)	2.5ml
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Phosphate Buffer Saline	to 250ml
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Store at 4°C

Chemiluminescence Buffer B

Casein blocking buffer (Genosys)	12.5ml
----------------------------------	--------

Tween 20	1.25ml
----------	--------

Sodium azide (10% stock soln.)	2.5ml
--------------------------------	-------

SSC (20 x stock soln.)	125ml
------------------------	-------

Water	to 250ml
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Store at 4°C

TTA wash buffer (100x stock)

Tween 20	50ml
Sodium azide	100g
1M Tris/HCl, pH 7.5	to 1L

Dilute 1:10 for storage in PCR room

Dilute 1:10 stock 1:10 for use

Store at room temperature

1.5M NaOH

NaOH	1.5g
Water	to 25ml

Store for up to 2 weeks

Dilute 1:10 for use.

Store dilution (0.15M) for up to 2 days

Store at room temperature

SSC (20 x stock)

NaCl	175.32 g
tri-sodium citrate (2.H ₂ O)	88.23 g
Water	to 1L

Store at room temperature

Reagents and consumables

rTth polymerase, 5 x reaction buffer,

Na acetate Perkin-Elmer, Cat. No. N808-0178

dNTPs Pharmacia, Cat. No. 27-2035-01

Oligonucleotide primers Oswell

Alkaline phosphatase labelled probes Oswell

Silica gel Sigma, Cat No. S 5631

Guanidine thiocyanate Sigma, Cat. No. G6639

Nuclease free water Promega, Cat. No. P1194

Casein blocking buffer Genosys, Cat. No. SU-07-250

Streptavidin Sigma, Cat. No. S4762

Lumiphos₅₃₀ Lumigen

Other chemicals and reagents should be Analar or molecular biology grade

RNA extraction vials Sarstedt, Cat No.72-692-005

Filter tips Gilson (Anachem), Cat. No. RT96F4, RT200F3

Straptavidin Coated Microtitre wells Thermo Life-Sciences

Plate sealers ICN, Cat. No.7740005

Primer and probe sequences

IEP1B

AGT TGG AGG ACA TCA AGC AGC CAT GCA AAT

IEP2A-bio

Bio - TGC TAT GTC ACT TCC CCT TGG TTC TCT

IEL1-AP

AAT GGG ATA GAT TGC ATC CAG T

Qa-AP

ACA GTG TAG ATA GAT GAC AGT C

Qb-AP

ATG CAA GGT CGC ATA TGA GTA A

Qc-AP

ATA AGC ACG TGA CTG AGT ATG A

APPENDIX 3. PUBLICATIONS ARISING AND RELATED TO THE THESIS

Papers

1. Sadiq ST, Copas AJ, Johnson AM. Factors Associated with Gonorrhoea in men aware of being positive for HIV infection: Case control Study. *BMJ* 317:1052-1053 1998.

Many gay men with STIs attending genitourinary medicine clinics are aware of being positive for HIV infection. This study showed that younger age at diagnosis of HIV infection and higher CD4⁺ T-cell counts were significantly associated with the acquisition of gonorrhoea and prompted the question of whether successful combination therapies might increase the risk of acquiring STIs and increasing viral load levels in semen.

2. Sadiq ST; Taylor S; Kaye S; Bennett J; Johnstone R; Byrne P; Copas AJ; Drake SM; Pillay D; Weller IVD. The effects of antiretroviral therapy on HIV-1 RNA Loads in Seminal Plasma in HIV positive patients with and without urethritis. *AIDS* 2002, 16:219-225.

3. Taylor S, Sadiq ST, Weller IVD, Kaye S, Workman J, Cane P, Bennett J, Cane PA, Copas A, Drake SM and D. Pillay. Drug-resistant HIV-1 in the semen of men receiving antiretroviral therapy with acute sexually transmitted infections. *Antiviral Therapy* 8:1-198

4. Smith R, Copas AJ, Prince M, George B, Walker AS, Sadiq ST. Poor sensitivity and consistency of microscopy in the diagnosis of low grade non-gonococcal urethritis. *Sexually Transmitted Infections*. 2003;79:487– 490

This study highlighted the considerable observer variation association with diagnosis of NSU, and influenced our decision in the definition of the condition in one of our studies.

5. Sadiq ST, Taylor S, Copas AJ, Bennett J, Kaye S, Drake SM, Kirk S, Pillay D, Weller IVD. The effects of urethritis on seminal plasma HIV-1 RNA loads in homosexual men not receiving antiretroviral therapy. *Sexually Transmitted Infections* 2005; 81(2).

6. Sadiq ST, McSorley J, Copas AJ, Bennett J, Edwards SJ, Kaye S, Kirk S, French P, Weller IVD. The effects of early syphilis on CD4 counts and HIV-1 RNA viral loads in blood and semen. *Sexually Transmitted Infections* (in press).

7. Sadiq ST, Copas AJ, Weller IVD. Research involving semen donation is feasible and acceptable among gay men co-infected with HIV-1 and genital infections. *Sexually Transmitted Infections* (submitted for publication).

Abstracts and oral presentations

Sadiq ST, Taylor S, Copas AJ, Bennett J, Kaye S, Drake SM, Kirk S, Pillay D, Weller IVD. The effects of gonococcal, chlamydial and non-specific urethritis on seminal plasma HIV-1 RNA loads in the UK. In abstracts of the 11th Conference on Retroviruses and Opportunistic infections, San Francisco, February 2004 (poster).

Sadiq ST, McSorley J, Copas AJ, Bennett J, Edwards SJ, Kaye S, Kirk S, French P, Weller IVD. The effects of early syphilis on CD4 counts and HIV-1 RNA viral loads in blood and semen. In Abstracts of the ISSTD Congress, Ottawa, July 2003 (poster).

ST Sadiq, S Taylor, S Kaye, J Workman, P Cane, J Bennet, A Copas, S Drake, I Weller, D Pillay. Multi-Drug Resistant HIV-1 in the Semen of Men with Acute Sexually Transmitted Infections. In abstracts of the 8th Annual Conference of the British HIV Association, University of York April 2002, (oral presentation).

S.Taylor, T. Sadiq, S.Kaye, J. Workman, P.Cane, J. Bennet, R. Johnstone, P.Byrne, A. Copas, S. Drake, I.Weller, D. Pillay. Prevalence of HIV-1 Drug Resistance in Semen of Patients on HAART with Acute Sexually Transmitted Infections. In: Abstracts of the 9th Conference on Retroviruses and Opportunistic Infections. February 2002, Seattle, USA (poster).

ST Sadiq; S Taylor; S Kaye; J Bennett; D Pillay; I Weller. The effects of antiretroviral therapy on HIV-1 RNA Loads in Seminal Plasma in HIV positive patients with and without urethritis. In abstracts of The 1st IAS Conference on HIV Pathogenesis and Treatment. July 2001, Buenos Aires, Argentina (poster presentation).

Sadiq ST, Kaye S, Bennett J, Johnstone R, Copas AJ, Weller IVD. Changes in HIV-1 RNA loads in seminal plasma during episodes of chlamydial, non-Gonococcal and gonococcal urethritis in patients on Highly Active Antiretroviral Therapy (HAART).

In abstracts of The Joint meeting of the ASTDA & the MSSVD, Baltimore, USA, May 2000 (oral presentation).

Sadiq ST, Copas AJ, Johnson MA. Factors Associated with Gonorrhoea in Patients Aware of their HIV Positive Status: A Case control Study. In abstracts of The Spring Meeting of the British HIV Association, March 1998 (oral presentation).

APPENDIX 4. DECLARATION OF WORK PERFORMED BY THE CANDIDATE

The vast majority of work presented in this thesis was done by the candidate over a period of four years. The contributions of others are detailed in the acknowledgements section. The candidate made the following contributions to this work:

Study design and preparation. Conception and design of all studies described in this thesis and preparation of all protocols and submissions to ethics committees.

Recruitment of subjects and collection of data. Recruitment of subjects and collection of all clinical data for all studies apart from four patients in the study on urethritis and antiretroviral therapy described in chapter 3 (one of whom had resistance data presented in chapter 4) and 18 patients recruited to the study in presented in chapter 5. I was responsible for the day-to-day running of all projects, working with clinical and nursing staff to ensure maximal publicity and awareness within the clinic.

Laboratory work. Measurement of blood and semen HIV-1 RNA loads, including HIV-1 RNA extraction, amplification and assay for all patients in the two studies presented in chapters 5 and 6.

Data analysis and interpretation. Collation of all data and entry into computer database apart from the data presented in chapter 4. I performed statistical analysis on the comparison of two viral load measurements (presented in chapter 2) and interpreted the statistical analysis for all studies where done.

Dissemination of information. Presentation of data at national and international conferences. Writing and revising all manuscripts for publication.

APPENDIX 5. ACKNOWLEDGEMENTS

I express my deepest thanks and gratitude to my supervisor, Professor Ian Weller, for supporting me in an academic career and facilitating the completion of this work. Professor Weller gave vital input and advice into the design and interpretation of results of the studies, critically reviewing all chapters and publications arising.

My second supervisor, Dr Steven Kaye, then senior scientist in Virology at UCL was a source of constant support and advice. Dr Kaye and Ms Julie Bennett (scientist) did viral load analysis on the patients using the in-house assay presented in chapters 2 and 3. Dr Deenan Pillay provided the archived semen samples with known HIV-1 loads that were analysed by the in-house assay (chapter 2) and was readily available to provide advice on projects. Julie gave me laboratory training required for performing HIV-1 RNA analysis, which I used in the studies presented in chapters 5 and 6. Stuart Kirk performed genotyping of virus performed described in chapter 6. Dr Andrew Copas (Lecturer in statistics) helped in the design of studies and performed much of the complex analysis presented in this thesis. Andrew also critically reviewed publications arising from this thesis.

Dr Stephen Taylor, from Birmingham Heartlands hospital, did the resistance testing on the four subjects described in chapter 4 and collaborated with me in two of the studies. Stephen provided samples from 22 patients in all from Birmingham Heartlands Hospital. Dr John Mcorley and Dr Patrick French helped in the design of the retrospective study on syphilis (chapter 6) and Dr Sarah Edwards assisted in data entry for this project. Martin Prince, senior MLSO, Microbiology, UCH and Patrick Pearson, Centre for sexual health & HIV research for assistance in identifying cases of early syphilis.

The sexual transmission of HIV-1 is largely dependent on the nature of the virus in genital secretions and studies of the virus in the genital compartment are important in the effort to control the HIV-1 pandemic. These studies are difficult to undertake and require the cooperation of staff and patients. Thus sincere gratitude is due to all staff at the Mortimer Market Centre, Camden Primary Care Trust who constantly sought to promote the projects and to the patients who were able to overcome significant embarrassment in order to participate, because they wanted to help.

Finally a special thanks to my wife, Patricia, whose love and commitment to me through difficult times has been absolute and my children, Sean, Fintan, Safia and Aneesa who put up with Dad not being there on many occasions.

It is with humility that I recognise that many people, some mentioned and others perhaps even unknown to me, have made this thesis and series of studies possible.